UNZIPPED MEDIATES AXON GUIDANCE OF *DROSOPHILA MELANOGASTER* MUSHROOM BODIES THROUGH NEURON-NEURON AND NEURON-GLIA INTERACTIONS

by
Bahadır Çağrı Çevrim
B.S., Molecular Biology and Genetics, Boğaziçi University, 2014

Submitted to the Institute for Graduate Studies in
Science and Engineering in partial fulfilment of
the requirement for the degree of
Master of Science

Graduate Program in Molecular Biology and Genetics
Boğaziçi University
2016

to whomever loved but could not coalesce…

ACKNOWLEDGEMENTS

I would like to thank all of my professors, collegues, friends and family for their guidance, patience and support.

This project was supported by the funds of TUBITAK project number 113T086.

ABSTRACT

UNZIPPED MEDIATES AXON GUIDANCE OF *DROSOPHILA MELANOGASTER* MUSHROOM BODIES THROUGH NEURON-NEURON AND NEURON-GLIA INTERACTIONS

Drosophila mushroom body (MB) neuropil is the regulatory center for learning, memory, and many different behaviors. Besides its functional role, MB is a great model to investigate axon guidance dynamics. The MB consists of three different types of neurons that have very similar yet distinctive axon projection patterns. The morphology and the cellular mechanisms of MB development are well characterized and many genetic tools have been developed that allow researchers to address different questions. The requirement for neuron-neuron interactions during MB axon guidance has been relatively well characterized (Siegenthaler *et al*., 2015), however the role of neuron-glia interactions during this process are still unresolved. Here, we focused on the role of *unzipped* (*uzip*) in MB axon guidance. Initial experiments with mutant lines revealed that *uzip* is required for proper MB axon guidance. Knockdown experiments in different neuron populatins revealed that expression of Uzip by MB neuron subtypes is required for the interactions between those cells to mediate axon guidance. Apart from neuronal Uzip, glia-specific loss-of-function (LOF) and molecular rescue experiments revealed that Uzip expression glial cells is also required for proper MB development. Taken together, functional analyses experiments revealed that during MB axon guidance Uzip is not only involved in previously defined neuron-neuron interactions but also mediating previously unknown neuron-glia interactions. Additionally, Uzip signaling that navigates MB axons include axon guidance molecules *nrg*, *sema-1a*, *CadN* and *drl*. All of those molecules were previously shown to be regulating MB axon guidance and this study contributes by both revealing the molecular networks in more detail and also bringing glia into the game.

ÖZET

DROSOPHİLA MELANOGASTER MANTARSI YAPILARININ GELİŞİMİNİN UNZIPPED TARAFINDAN, NÖRON-NÖRON VE NÖRON-GLİYA ETKİLEŞIMLERİ ÜZERİNDEN KONTROLÜ

Drosophila mantarsı yapıları (MY) öğrenme, hafıza ve pek çok farklı davranışın düzenlendiği nöropildir. İşlevsel rolünün yanısıra, MY axon gelişim dinamiklerini araştırmak için önemli bir modeldir. MY birbirine benzerlikleri olsa da farklı akson yöneliminine sahip üç farklı tip nörondan oluşur. MY gelişıminin morfolojisi ve gelişim esnasındaki hücresel dinamikler detaylı olarak tanımlanmıştır ve geliştirilen pek çok genetik araç değişik araştırmalar yapmayı mümkün kılmaktadır. MY gelişimi esnasında nöron-nöron etkileşimlerinin rolü görece iyi tanımlanmıştır (Siegenthaler ve ark., 2015), fakat nöron-gliya etkileşimlerinin rolü hala bilinmemektedir. Bu araştırmada *unzipped*'in (*uzip*) MY axon yönelimdeki rolü araştırılmıştır. Mutant bireyler ile yapılan ilk deneyler *uzip*'in MY akson yönelimi için gerekli olduğunu göstermiştir. Değişik nöron popülasyonlarında *uzip* ifadesinin susturulmasıyla, MY nöron alt türleri tarafından Uzip ifadesinin bu nöronların birbirleriyle etkileşimi için zaruri olduğu gözlemlenmiştir. Bunun yanısıra, gliyalara özgül ifade susturma deneyleri sonucunda gliya hücrelerinin de MY akson yönelimi için gerekli olduğu görülmüştür. Tüm işlevsel analizler birlikte ele alındığında, Uzip'in yalnızca daha önceden bilinen nöron-nöron etkileşimlerinde değil; bu ilk defa bu çalışma esnasında ortaya çıkartılan nöron-gliya etkileleşimlerinde de önemli rolü olduğu görülmüştür. Ayrıca, MY aksonlarının yönelimini kontrol eden Uzip sinyalizasyonu, daha önce tanımlanmış akson kılavuz kılavuz moleküllerinden *nrg*, *sema-1a*, *CadN* ve *drl*'yi de içermektedir. Bu moleküllerin MY akson yönemlimini düzenledıği daha önceden gösterilmir ve bu çalışma hem bu etkileşim ağlarının daha detaylı anlaşılmasını sağlamakta hem de gliyaları da bu mekanızmya dahil etmektedir.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS iv
ABSTRACT v
ÖZET vi
TABLE OF CONTENTS vii
LIST OF FIGURES x
LIST OF TABLES xii
LIST OF ACRONYMS/ABBREVIATIONS xiii
1. INTRODUCTION 1
    1.1. Axon Guidance 1
        1.1.1. Axon Guidance Molecules 2
        1.1.2. Role of CAMs in Axon Guidance 2
    1.2. Glia 4
        1.2.1. Role of Glia in Axon Guidance 5
    1.3. Mushroom Bodies 6
        1.3.1. Development of *Drosophila* Mushroom Bodies 7
        1.3.2. Role of Glia in Mushroom Body Development 9
    1.4. Unzipped: a New Player in Neuron-Glia Interactions 11
2. AIM OF THE STUDY 12
3. MATERIALS AND METHODS 13
    3.1. Biological Methods 13
    3.2. Chemicals and Supplies 14
        3.2.1. Antibodies 14
        3.2.2. Chemical Supplies 14
        3.2.3. Buffers and Solutions 15
        3.2.4. Embedding Media 15

3.2.5. Disposable Labware ... 16
3.2.6. Equipment ... 16
3.3. Experiments for Localizing Uzip Expression ... 17
3.3.1. Expression Analysis of Uzip ... 17
3.3.2. Uzip LOF experiments ... 17
3.3.3. Molecular Rescue Experiments ... 18
3.3.4. Genetic Interaction Analyses ... 19
3.4. Histological Techniques ... 19
3.4.1. Immunohistochemistry ... 19
3.4.2. Visualization and Image Processing ... 20
4. RESULTS ... 21
4.1. Analyses of the Expression Pattern of Unzipped Around Mushroom Bodies ... 22
4.2. Analyses of Mushroom Body Morphology of *uzip* Mutants ... 24
4.3. Analyses of the Effect of Neuronal KD of Uzip on MB Development ... 27
4.3.1. Testing the Specifity and Efficiency of Different UzipRNAi Lines ... 29
4.3.2. Slincing Uzip Expression in Mushroom Body Neuron Subtypes ... 30
4.4. Analyses of the Effect of Neuronal KD of Uzip on EB Development ... 32
4.5. Analyses of the Effect of Glial Knock-Down of Uzip on MB Development ... 35
4.5.1. Pan-Glial Uzip Loss-of-Function ... 35
4.5.2. Analyses of the Role of Uzip in TIFR – MB Interactions ... 37
4.5.3. Assesing the Importance of Uzip Expression by Ensheathing and Astrocycte-like Glia on Mushroom Body Development ... 38
4.6. Molecular Rescue Experiments ... 40
4.6.1. Rescue with Uzip BAC Transgenic Lines ... 40
4.6.2. Neuron-Specific Molecular Rescue Experiments ... 41
4.6.3. Glia-Specific Molecular Rescue Experiments ... 43
4.6.4. Identification of Genetic Interaction Network of Uzip During Mushroom Body Development ... 43
5. DISCUSSION ... 52
5.1. Uzip is Required for Neuron-Glia Interactions During Development ... 53
5.1.1. Uzip is Expressed by Glia Associated with Mushroom Bodies ... 53
5.2. Uzip is Required for Path Decision of Kenyon Cell Axons ... 55

5.3. Mushroom Body Neurons Need to Express Uzip for Proper Axon Guidance.... 57
5.4. Uzip Mediates Interactions Between MB Neuron Subtypes.............................. 58
5.5. Glial Uzip is Required for Mushroom Body Axon Guidance............................ 60
5.5.1. Uzip LOF in MB Associated Glia does not Effect MB Development.. 61
5.5.2. Uzip Expression by TIFR Glia is Required for TIFR-MB Interaction . 62
5.6. Uzip Expression in both Neuron and Glia is Required for MB Axon Guidance 63
5.6.1. Neuronal Uzip is Enough to Rescue MB Phenotypes........................... 64
5.6.2. Only Glial Uzip Expression is not Sufficient to Rescue MB Axon Guidance Defects without Neuronal Uzip 65
5.7. Uzip Signaling During MB Axon Guidance Includes Previously Identified Axon Guidance Molecules 66
5.7.1. *Uzip* is Involved in the Same Genetic Interaction Network with *nrg* and *sema-1a* to Control MB Axon Guidance.. 67
5.7.2. *Uzip* Signaling During the MB Axon Guidance does not Include *Wnt5* but Its Receptor *drl* 67
5.7.3. *CadN* is Involved in *uzip* Signaling During MB Development 68
5.7.4. Other Genetic Interaction Candidates 69
6. CONCLUSION.. 70
REFERENCES 71

LIST OF FIGURES

Figure 1.1. Long and short range cues are directing the guidance of the axons........3

Figure 1.2. Morphology of the adult *Drosophila* mushroom bodies. 7

Figure 1.3. Transient Interhemispheric Fibrous Ring...10

Figure 3.1. Crosses for Uzip localization experiments. 17

Figure 3.2. Crosses for Kenyon cell specific KD experiments. 18

Figure 3.3. Crosses for cell type specific molecular rescue experiments. 18

Figure 3.4. Crosses for genetic interaction analyses. 19

Figure 4.1. Localization of Uzip around mushroom body neuropil. 23

Figure 4.2. Analyses of mushroom body phenotypes in *uzip* mutants. 25

Figure 4.3. Quantification of mushroom body phenotypes in *uzip* mutants. 26

Figure 4.4. Analyses of MBs of *uzip* mutants at late larval stage. 27

Figure 4.5. Analyses the phenotypes caused by neuronal Uzip KD. 28

Figure 4.6. Quantification of MB phenotypes in MB specific KD experiments. 29

Figure 4.7. Assesing the specificty and efficiency of UzipRi lines. 31

Figure 4.8. MB neuron subtype specific KD experiments......................................33

Figure 4.9. Uzip KD in α / β pioneer neurons...34

Figure 4.10. Effect of Uzip KD on EB morphology. 35

Figure 4.11. Analyses of EB specific KD. 36

Figure 4.12. Analyses of glial Uzip loss-of-function experiements. 37

Figure 4.13. The role of Uzip on TIFR-MB interactions. 39

Figure 4.14. Analyses of Uzip requirement by MB associated glia. 40

Figure 4.15. Molecular rescue with *Uzip* BAC constructs. 41

Figure 4.16. Rescue of neuronal Uzip in the null mutant flies. 42

Figure 4.17. Rescue of glial Uzip in the mutant flies. 44

Figure 4.18. Quantification of MB phenotypes in glial rescue experiments. 45

Figure 4.19. Analyses of genetic interaction of *nrg* and *CadN* with *uzip*...............47

Figure 4.20. Analyses of genetic interaction of *sema-1a* and *drl* with *uzip*. 48

Figure 4.21. Anayses of genetic interaction of *fasii*, *wnt5* and *Dscam* with *uzip*....49

Figure 4.22. Analyses of genetic interaction of *netrinA*/*B, trio* and *Abl* with *uzip*..51

LIST OF TABLES

Table 3.1. Fly lines used in this study. 13

Table 3.2. Antibodies used in this study. 14

Table 3.3. Chemicals used in this study. 15

Table 3.4. Buffers and Solutions used in this study. 15

Table 3.5. Disposable labware used in this study. 16

Table 3.6. Equipment used in this study. 16

LIST OF ACRONYMS/ABBREVIATIONS

BAC	Bacterial artificial chromosome
CAM	Cell adhesion molecule
CNS	Central nervous system
EB	Ellipsoid body
GFP	Green fluorescent protein
GOF	Gain of function
KO	Knock out
LOF	Loss of function
MB	Mushroom body
OSN	Olfactory sensory neuron
PR	Photoreceptor
RNAi (Ri)	RNA interference
TIFR	Transient interhemispheric fibrous ring
UAS	Upstream activating sequence
Uzip	Unzipped
VNC	Ventral nerve cord

1. INTRODUCTION

The first known scientist who attributed the responsibility of certain behaviours, emotions and activities as well as mental well-being was Hippocrates around fourth century BC. This idea has been confirmed and improved over the centuries by numerous scientists. Finally, more than two millennia after this initial statement of Hippocrates, the amazing neuroanatomical studies of Santiago Ramón y Cajal and his identification and description of cellular structures of the central nervous system (CNS) had opened a whole new world.

The neurons are highly divergent in their anatomy but generally have three main compartments namely the soma, dendrite, and axon. Each neuron has to connect with other neurons in precise patterns. Connections are established between dendrites and axon terminals and these connections are called synapses. Considering an average human brain that has about 100 billion neurons and approximately 50 times more glial cells (Herculano-Houzel, 2012), this means an enormous neural network. Every single neuron has to grow and project its axon through this large amount of cells and find their correct synaptic targets for the orchestrated function of the brain. The mechanical and molecular mechanisms in which this axon guidance process is finely tuned have been investigated quite intensively yet there is a lot to discover.

1.1. Axon Guidance

The sizes of neurons vary between a couple of micrometres to tens of meters (Smith, 2009). Most of this length is due to the elongated axons that develop after the cells are born. So, an axon has to travel large distances compared to the size of its soma. Anatomical investigations revealed that every axon has a very well defined tract and most of these tracts are very well represented in different individuals of a species (Weiss, 1934).

Current data indicate that axon guidance mechanisms are shared between most neurons, however the fine-tuning of individual mechanisms and interactions between them results in different projection patterns of different axons. Consequently, understanding how an individual axon or a group of axons find their path is the initial step of understanding complex neural wiring in detail. Initial studies to identify cellular mechanisms in CNS development revealed that developing axons are growing along pre-existing axonal tracts (Goodman and Shatz, 1993). So, the question of how an axon decides what signals to follow and how to follow them has been the central question for many scientists since then.

1.1.1. Axon Guidance Molecules

The developing axons have specialized tips with many spiky extensions. This flattened; glove-like structures are called growth cones. The spiky protrusions of the growth cones have a different actin composition than the rest of the cell, which enables them to extend and retract in a very dynamic manner. The extension and retraction movements are regulated by the long and short-range guidance cues that are perceived by the molecules on the membrane of the cell (Figure 1.1). The molecules that serve as cues in the substrate of the axon and are required to guide the developing axon are collectively called axon guidance molecules (reviewed in Tamariz and Varela-Echavarría 2015). Some of the axon guidance molecules are expressed by the growing neuron and others by the neighbouring cells. The ones expressed by the cell itself are enriched on the growth cones and they generally interact with axon guidance molecules in the environment. Thus, proper targeting of an axon is the result of a fine tuned production of axon guidance molecules both by the navigating axon and the cells in the substrate, and the interplay between these two different pools of molecules (Vitriol and Zheng, 2012).

1.1.2. Role of CAMs in Axon Guidance

In most of the cases, the initial and main driving forces of axon guidance are attractive and repulsive long-range chemical cues. Throughout development, axons need to receive physical support from their environment and this support is provided by cell-to-cell

or cell-to-extracellular matrix adhesions. CAMs are mediating those adhesions and regulation of CAM expression and regulation of their activity and turnover is directly affecting the motility of the developing axon (Kaplan *et al*., 2014).

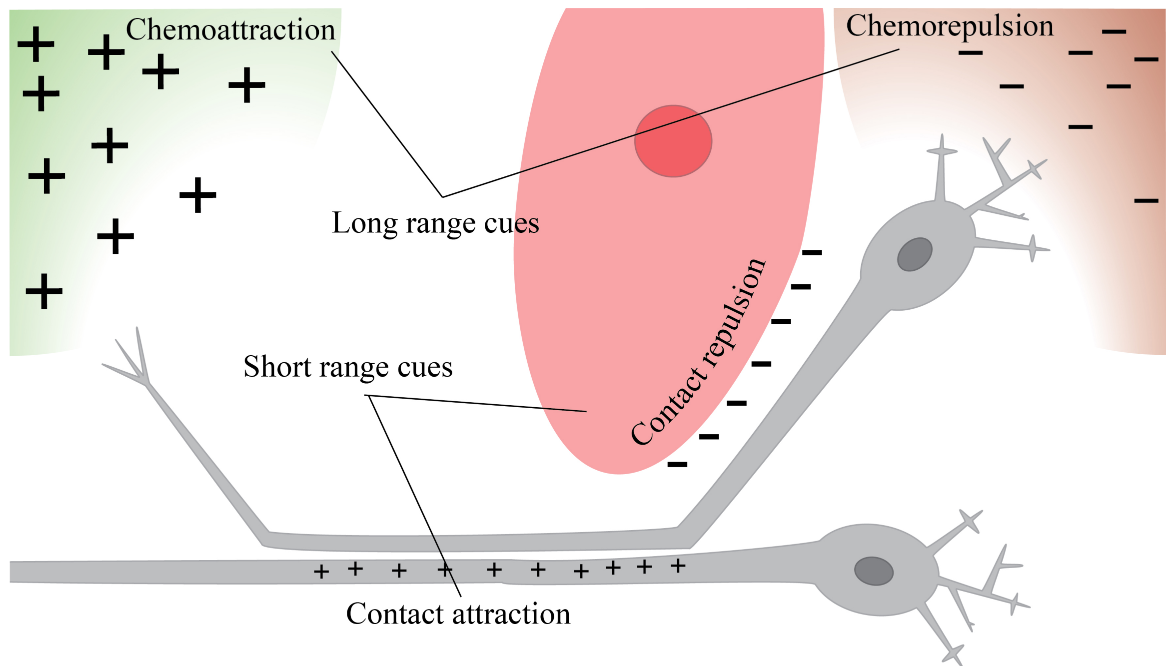

Figure 1.1. Long and short range cues are directing the guidance of the axons (adapted from Kolodkin and Tessier-Lavigne, 2011).

Besides the mechanical support for the cells, some CAMs regulat the behaviour of the growing axon by activating secondary signalling mechanisms upon binding. In this way, the cells are able to regulate their activities according to the identity of their substrates. Thus, axons can bundle together and stay like that and terminate the growth when they reach a certain size or make contact with a certain target (Pollerberg *et al*., 2013). There are several CAM families, some of which are conserved very well throughout evolution whereas some are clade-specific. However, the mechanism in which CAMs exert their function has a lot of similarities even though the individual proteins are very different (Jacobs and Goodman, 1989).

1.2. Glia

Development of more complex cognitive and motor behaviours has been mainly attributed to the increased diversity and more complex functional organisation of neurons, for a long time. However, in the recent two decades, scientists started to realize that the second major cell type of the CNS – glia – has very important roles in the development and proper functioning of the brain. For a long time, glial cells have been considered only as supporting cells for neurons and not many studies focused on them. This was mainly because they were not as exciting as their electrically-active neighbours and there were not many tools to observe them (Villegas *et al*., 2003). One of the most striking observations that gave glia research a boost was that the ratio of glia to all cells in CNS was found to be ~10% in flies and ~90% in humans (Doherty *et al*., 2009). This observation is raising the question if the number of glia in the brain correlates with the complexity of brain function?

Since the knowledge about glia is still very limited, it is very hard to classify them according to their function. In mammals four main glial cell types were classified according to their morphology; astrocytes, oligodendrocytes, microglia, and Schwann cells. *Drosophila* also has four main classes of glia, namely cortex, neuropil, surface, and peripheral glia, and they exhibit many similarities both in their morphology and function to the mammalian glia (Freeman and Doherty, 2006). Since the diversity, anatomy and the function of glia is conserved very well *Drosophila* serves as an excellent model to study glial biology.

Surface glia generate a cellular layer that wraps the entire brain like a sheath. One subtype of surface glia, subperineural glia, generates septate junctions and gap junctions around the brain surface and generate the blood-brain barrier of the *Drosophila* brain. The other subtype, perineural glia, forms the outer-most layer that surrounds the brain. Even though these cells are thought to be contributing to the function of the blood-brain barrier, their function is unknown (Hartenstein, 2011).

Cortex glia are located within the cell cortex and they have a very small cell body and nucleus. Those glial cells generate a capsule-like structure for individual neuronal

somata and neuroblasts and form a scaffold. In contrast to their small cell body, each cortex glia can encapsulate several neuronal cell bodies (Pereanu *et al*., 2005).

Neuropile glia comprise the most diverse class of glia in *Drosophila* brain. This type of glia are generally located between the cortex and the neuropile. Especially around the central complex a high concentration of cell bodies is present. One subtype, ensheathing glia, ensheaths the neuropile and insulates it from the environment (Takeshi Awasaki *et al*., 2008). Another type, astrocyte-like glia, infiltrate the neuropil and engulf the neuronal cell debris (Hakim *et al*., 2014). Another important subtype, wrapping glia, ensheath the neurites in the periphery (Hartenstein, 2011).

Even though they are CNS derived, peripheral glia are function in the peripheral nervous system. They insulate individual axons and function as the *Drosophila* counterpart of Schwann cells (Hartenstein, 2011).

1.2.1. Role of Glia in Axon Guidance

As explained previously, neurons need to receive signals from their environment to to project their axons. Around four decades ago, it was revealed that the signals are coming from “guidepost cells” in the brain (Bate, 1976). These guidepost cells mediate axon guidance by mediating attraction or repulsion of the axons through direct contact or by secreting chemical compounds. Almost three decades after the definition of guidepost cells, it was shown that axons cannot project when glial cells are absent in the CNS (Hidalgo and Booth, 2000). Shortly after the same phenomenon was also observed for the development of the peripheral nervous system (Sepp *et al*., 2001).

There are several examples of neuron-glia interaction during axon guidance, but relatively well-defined examples include the development of visual and olfactory sensory neuron projection. Olfactory sensory neurons (OSN) project into the brain as fascicles and the fascicles contact with sorting-zone glia just before entering the antennal lobe. This contact ceases the axonal growth and leads to morphological changes in the growth cone.

After this contact, the fascicles disentangle so that each OSN projects to different glomeruli according to their identity (Tucker *et al*., 2004).

During visual system development the initial axon projection of photoreceptor (R) cells extend their axons from eye disc to the brain through the optic stalk. And in the absence of retinal basal ganglia, R axons fail to extend to the optic stalk (Rangarajan *et al*., 1999). In later stages of R cell development glia are also controlling the projection of axons to the lamina (Edenfeld *et al*., 2005).

1.3. Mushroom Bodies

Mushroom Bodies (MBs) are higher brain centers located in the protocerebral brain segments of *arthropods*. Even though the morphologies vary between different species, all MBs consist of intrinsic neurons called Kenyon cells. Those cells have tiny soma and project their axons in parallel to each other to generate the lobular structure of the MB (Strausfeld *et al*., 2009).

The most prominent role of insect MBs is being the center of olfactory learning and memory. Lesion experiments performed in different species including *Drosophila melanogaster* and *Apis mellifera* shows that MBs are required for new memory formation and retrieval of existing memories (reviewed in Heisenberg 2003). Apart from that, *Drosophila* studies revealed that MBs are important for regulation of other behavioural processes such as temperature preference behaviour (Hong *et al*., 2008), odor attraction (Y. Wang *et al*., 2003), gustatory associative learning (Masek and Scott, 2010) and habituation (Cho *et al*., 2004). From these functional aspects insect MBs are compared to vertebrate cerebellum-like structures (Farris, 2011).

In terms of structure and anatomy of the MBs the *Drosophila* MBs are the best studied. Each MB consist of three different types of Kenyon cells that are ~2000 cells in total. The cells bodies of these cells reside in the posterior-dorsal cortex and project their dendrites anteriorly to form the calyx. The initial axon segments of all three types of neurons generate the peduncle and further axon segments of γ neurons form the medial γ

lobe. The other two types of neurons α'/β' and α/β each generate vertical α' and α lobes and medial β' and β lobes, respectively (Lee *et al*., 1999) (Figure 1.2).

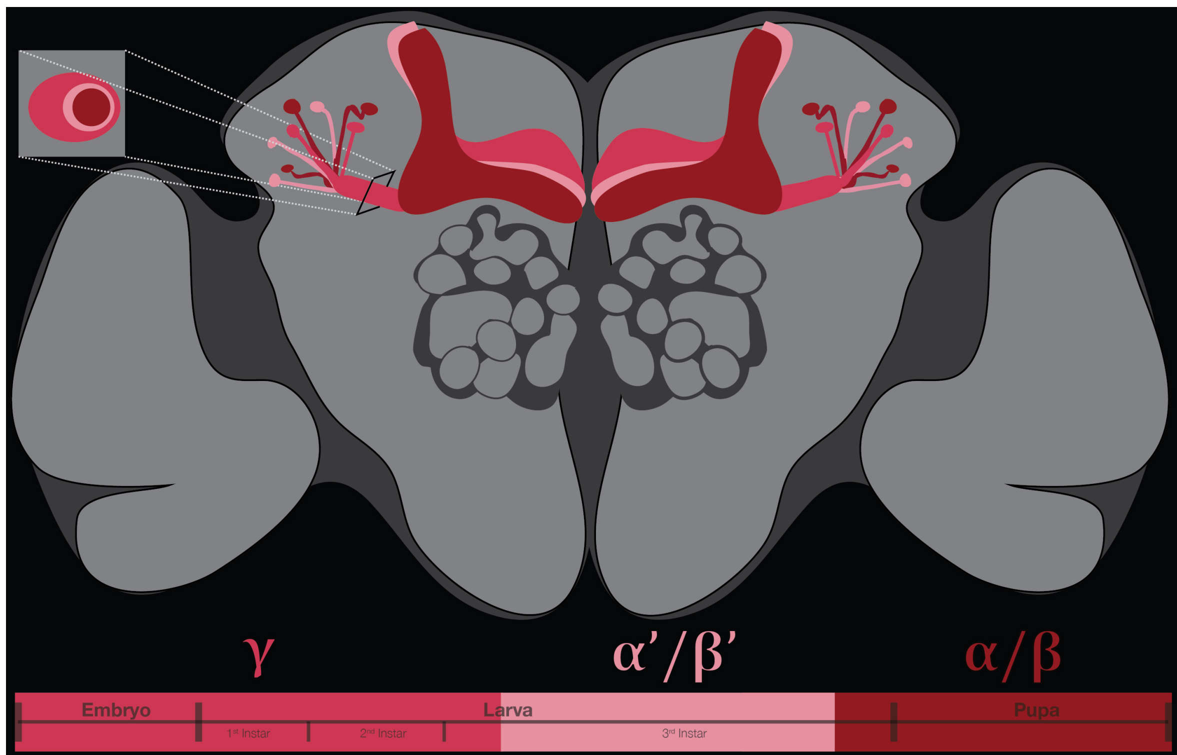

Figure 1.2. Morphology of the adult *Drosophila* mushroom bodies.
The MB (colored region) is a neuropil structure located in the dorsal part of the brain. Soma of all Kenyon cells reside at the posterior-dorsal cortex and project their axons through the peduncle. At the end of the peduncle each neuron subgroup generates distinct lobes. The bar shows the sequence of generation of different MB neuron subtypes. A transverse section through the peduncle shows that older neurons reside in the outer layers of the peduncle whereas young ones are located in the centre.

1.3.1. Development of *Drosophila* Mushroom Bodies

Drosophila MBs are generated by four neuroblasts (MBNB). At the embryonic stage those MBNB cells express distinct combinations of transcription factors, including *ey*, *rx*, *svp,* and *dac*. At embryonic stages MBNB produce intrinsic and non-intrinsic neurons, however the non-intrinsic neurons do not participate in the MB formation.

Initially it was suggested that MBNBs produce both neuronal and glial cells (Lee *et al*., 1999), however a later and more detailed study proved that MBNBs produce only neuronal cells (Kunz *et al*., 2012).

Each one of the MBNB cells give rise to a specific cell lineage that includes all three types of MB neurons. The size of the lineages is not adjusted through apoptosis of differentiated neurons as it happens for many NB lineages; adjustments happen rather through regulation of the mitotic activity of the NBs (Kunz *et al*., 2012).

Generation of three different MB neurons from each one of the MBNBs occurs in a temporal sequence. Throughout the embryonic and early larval stages the only MB neuron type generated are γ neurons. When the animal reaches the 3rd instar larval stage, MBNBs make a switch and start to generate α'/β' neurons and this continues until the end of larval stages. At the end of the larval stages, the last type of MB neuron, α/β, starts to be generated. Initially, a small population of pioneer neurons called α/βp neurons are produced. However, these neurons are morphologically indistinguishable from the rest of the α/β neurons. MBNB activity ceases with the end of the pupal stage and the numbers of MB neurons are fixed after this stage (Kunz *et al*., 2012; Lee *et al*., 1999) (Figure 1.2).

Each MB neuron subtype has a different axonal projection pattern. After they are born, γ neurons project their axons anterovertically and generate the peduncle. When the axon tips reach the end of the peduncle they branch. One of the branches projects medially and the other dorsally, generating two distinct lobes. During the pupal stage, all of the γ neurons undergo axon pruning and the axons are shortened until the end of the peduncle, again. Following this pruning, the neurons project again, but without branching this time, generating a single medial lobe (Hakim *et al*., 2014; Lee *et al*., 1999).

The second type of MB neuron, α'/β', initially follows the same track as their antecedents. Every newborn axon enters the peduncle through the centre and it projects as it is encircled by the previously formed axon projections. So, the first α'/β' neurons project through the peduncle with the help of previously formed γ trajectories. Expression of CAM Nrg by both of the neuron types mediates their interaction (Siegenthaler *et al*., 2015). At

the end of the peduncle each α'/β' neuron bifurcates and generates two sister axon branches. One of the branches projects ventrally and generates the α' lobe, whereas the other projects medially and generates the β' lobe. The early born α'/β' lobe finishes its axonal development before the γ neurons prune (Lee *et al.*, 1999). Since the lobular structures are very similar and spatially in close proximity, it had been suggested that γ neurons serve as a guidance cue for these neurons. However, there is no data proving this hypothesis.

The latest born MB neurons, α/β, follow almost the same fate with the α'/β' neurons. Again their projection through the peduncle is mediated by NRG expression, and they generate two distinct lobes called α and β. These lobes are located anterior of the α' and β' lobes. The initial small population of α/βp neurons use the guidance cues from the α'/β' neurons that already have completed their projections. The later born neurons use these pioneer neurons and the α'/β' neurons as guidance cues. If the α' or β' lobe does not form correctly, the following α or β lobe cannot form (Bates *et al.*, 2010; Goossens *et al.*, 2011; Siegenthaler *et al.*, 2015).

A lot of axon guidance molecules are shown to be required for proper MB development. The transcription factors Ey, Toy, and Dac have been shown to be important for the MBNB proliferation and early development of MBs (M Kurusu *et al.*, 2000; Noveen *et al.*, 2000). Later studies focusing on the role of Rac and Rho GTPases in MB development showed that growth, guidance and branching of MB neurons are independent events with separate regulatory mechanisms and are required at different levels of signalling activity (Ng and Luo, 2004; Ng *et al.*, 2002). Apart from Nrg, CAM molecules Dscam (J. Wang *et al.*, 2002), CadN (Mitsuhiko Kurusu *et al.*, 2012), FasII (Fushima and Tsujimura, 2007) are also required for proper MB development.

1.3.2. Role of Glia in Mushroom Body Development

MBs have being used as a model to study axon guidance for a long time. There are many studies explaining the cellular mechanisms of MB development and requirement of different molecules and signalling pathways. However, most of these studies are focusing

on neuronal mechanisms and molecules required in neurons. Compared to the amount of knowledge gathered about MBs the insight about the role of glial cells in MB development and function is scarce.

There are two glial populations that are in close contact with the MB neuropil during development and at adult stages (Ou *et al*., 2016). The first one, astrocyte-like glia, has a crucial role in γ neuron pruning during the pupal stage. They initiate axon fragmentation of the axons and then clear the axonal debris (Hakim *et al*., 2014). The second glial population, ensheathing glia, is not known to be involved in MB development, but it is important for proper functioning and ultimately the regulation of different behaviours (reviewed in Zwarts, Van Eijs, and Callaerts 2015).

The only glial population that is directly involved in MB axon guidance are the transient interhemispheric ring (TIFR) glia. TIFR glia become visible at the late 3rd instar larval stage and are localized at the dorsal side of the midline. At early stages of the pupa, protrusions of these cells form a ring structure between the two brain hemispheres. As the name indicates, this structure is formed transiently between ~25h and ~50h after pupal formation. Its interaction is crucial for OSN to cross the midline. Besides, this structure is also interacting with MB neurons and adjusting their position relative to the midline. When TIFR is ablated, the lobular structure of MBs is lost and all MB neurons collapse on the midline (Hitier *et al*., 2000; Simon *et al*., 1998) (Figure 1.3).

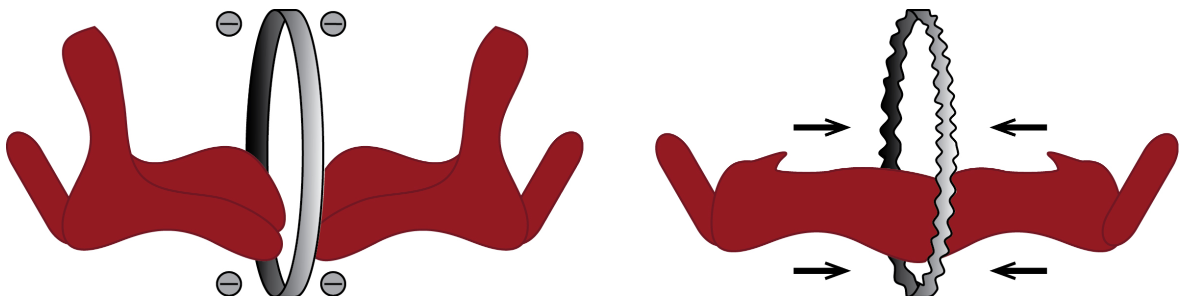

Figure 1.3. Transient Interhemispheric Fibrous Ring. In wild type condition TIFR is formed properly and generates a repellent effect on MB neurons so they do not pass the midline. When the TIFR structure is ablated, lobular structure of MBs is lost and all MB axons collapse on midline (adapted from Simon *et al*., 1998).

TIFR has four canals and OSN are crossing the midline through one of these canals, however the function of the other canals is not known. The CAM Nrg and the receptor tyrosine kinase Drl are required for proper TIFR formation and interaction between TIFR and the neurons (Chen and Hing, 2008; Simon *et al*., 1998).

1.4. Unzipped: a New Player in Neuron-Glia Interactions

Uzip encodes for a CAM that has no homology with any other CAMs and is conserved in arthropods. Uzip has two isoforms, one is GPI-anchored to the cell membrane and the other one is a cleaved and secreted form. In *Drosophila*, this protein is mainly expressed by glia and it mediates cell adhesion through homophilic binding. *Uzip* knock out (KO) lines do not exhibit any phenotypes but double KO lines with *wnt5* or *CadN* exhibit some axon guidance defects in embryonic ventral nerve cord (VNC). However, this interaction is solely genetic since there is no protein-protein interaction between Uzip and CadN (Ding *et al*., 2011).

A previous study conducted in our lab revealed that Uzip is expressed in the developing *Drosophila* retina. At late 3rd instar larval stage, a subset of R8 cells and some glial cells in the eye imaginal disc express. It was shown that some R7 cells display axon guidance phenotypes when uzip is overexpressed in all photoreceptor cells (Ece Terzioğlu-Kara, 2015).

Previous studies showed that Uzip is expressed in a single OSN subset at the adult stage and expressed by non-neuronal cell types in olfactory sensory organs, as well as glia associated with the olfactory bulbs in the brain. *Uzip* LOF results in OSN neurons unable to cross midline so the commissure of OSNs is lost (Selen Zülbahar, 2012) (Figure 1.6 B-C). Even though Uzip appeared to be expressed by a single OSN subtype, this commissure formation defect is observed in all OSN populations. Further LOF experiments revealed that knockdown of *uzip* in both neuronal and glial cell populations phenocopies the mutant phenotypes (Kaan Mika, unpublished). The TIFR structure is required for OSN commissure formation and *uzip* is shown to be required for proper TIFR formation (Kaan Mika, 2014; unpublished)

2. AIM OF THE STUDY

It is known that *uzip* is expressed mainly by glia but it is also expressed by neurons in the *Drosophila* central nervous system. In the embryonic VNC, Uzip mediates axon guidance in conjunction with CadN and Wnt5 (Ding *et al*., 2011). In the olfactory system, Uzip is required for OSNs to generate the commissure and cross the midline. Uzip expression from both OSN and TIFR glia, which mediates commissure formation, regulates this process (Kaan Mika, 2014).

MBs are one of the most prominent neuropil structures of insect brains. Preliminary studies revealed that Uzip is localized around this structure. This study has focused to understand the requirement of Uzip for *Drosophila* MB development. Considering the predominant glial expression pattern of Uzip, identification of the role of glia during MB axon guidance was also aimed. Additionally, the interaction partners of Uzip were aimed to be identified through genetic interaction analyses.

3. MATERIALS AND METHODS

3.1. Biological Methods

All *Drosophila* cultures were kept in 25°C and 80% humidity with a 12:12 dark and light cycle. Genesee Scientific Nutri-Fly™ BF fly food medium was prepared according to the instructor's manual and used for fly cultures. *Drosophila* stocks were transferred into new tubes with fresh medium once a month. Fly lines used in this study are listed in Table 3.1.

Table 3.1. Fly lines used in this study.

Transgene	**Inserted Chr.**	**Description**
AC783-Gal4	2	Enhancer trap line in which Gal4 has inserted in the *uzip* intron
elav-Gal4	X	Pan-neuronal Gal4 expression
OK107-Gal4	4	Gal4 expression in Kenyon cells
R15F02-Gal4	3	Gal4 expression in EB neurons
R16A06-Gal4	3	Gal4 expression in γ neurons
c305-Gal4	2	Gal4 expression in α'/β' neurons
c379-Gal4	2	Gal4 expression in α/β neurons
c708-Gal4	3	Gal4 expression in α/βp neurons
C442-Gal4	3	Gal4 expression in TIFR glia
repo-Gal4	3	Pan-glial Gal4 expression except midline glia
repo4.3-Gal4	2	Same as repo-Gal4
Alrm-Gal4	3	Gal4 expression in astrocyte-like glia
NP6520-Gal4	2	Gal4 expression in ensheathing glia
da-Gal4	3	Ubiquitous Gal4 expression
UAS-CD8::GFP	3	Membrane bound GFP expression under UAS control

Table 3.1. Mutant fly lines used in the study (cont).

UAS-UzipRi (TRiP)	3	Uzip RNAi construct expression under UAS control
UAS-UzipRi (KK)	2	Uzip RNAi construct expression under UAS control
UAS-UzipRi (GD)	3	Uzip RNAi construct expression under UAS control
UAS-Dcr2	2	Dcr2 expression under UAS control
UAS-Uzip	3	Uzip expression under UAS control
repo4.3-Gal80	3	Pan-glial Gal80 expression except midline glia
Uzip:mCherry	3	Uzip::mCherry fusion protein expression

3.2. Chemicals and Supplies

All chemicals and other supplies used in this study are listed under corresponding titles.

3.2.1. Antibodies

Antibodies used in this study are listed inTable 3.2.

Table 3.2. Antibodies used in this study.

Name	Antigen	Species	Dilution	Source
Primary Antibodies				
Anti-FasII	Fasciclin II	Mouse	1:200	DSHB (1D4)
Anti-GFP	GFP	Chicken	1:1000	Abcam (ab13970)
Secondary Antibodies				
Alexa 488	Chicken	Goat	1:250	Invitrogen
Alexa 647	Mouse	Goat	1:800	Invitrogen

3.2.2. Chemical Supplies

The chemicals used in this study are listed in Table 3.3.

Table 3.3. Chemicals used in this study.

Chemical	Manufacturer
Bovine Serum Albumin	Sigma-Aldrich, USA (A9647)
Paraformaldehyde	Sigma-Aldrich, USA (P6148)
Sodium Deoxycholate	Sigma- Aldrich, USA (30970)
Triton X-100	AppliChem, USA (A4975)

3.2.3. Buffers and Solutions

Buffers and solutions used in this study are listed in Table 3.4.

Table 3.4. Buffers and Solutions used in this study.

Buffer/Solution	Content
Formaldehyde Solution (16%)	160 g/l PFA, pH 7.4
PAXD	50 g BSA 3 g Sodium Dexoycholate 0.3% Triton X-100 in PBS
PBS	137 mM NaCl 2.7 mM KCl 10 mM Na_2HPO_4 1.8 mM KH_2PO_4
PBX3	0.3% Triton X-100 in PBS

3.2.4. Embedding Media

Following immunohistochemistry, all tissue samples were embedded in Vectashield Embedding Medium (Vector Laboratories, Inc).

3.2.5. Disposable Labware

Disposable labware used in this study are listed in Table 3.5.

Table 3.5. Disposable labware used in this study.

Material	Manufacturer
Micropipette tips	Grenier Bio-One, Belgium
Microscope slides and coverglass	Fisher Scientific, UK
Test tubes (0,5 – 1 – 2 ml)	Citotest Labware Manufactoring, China
Test tubes (15 and 50 ml)	Becton, Dickinson and Company, USA

3.2.6. Equipment

The equipments used in this study are listed in Table 3.6.

Table 3.6. Equipment used in this study.

Equipment	Manufacturer
Confocal Microscope	Leica Microsystems, Germany (TCS SP5-II)
Fluorescence Stereomicroscope	Leica Microsystems, Germny (MZ16FA)
Incubator	Weiss Gallencap, USA (Incubator Plus Series)
Inverted Microscope	Zeiss, USA (Axio Observer, ZI)
Micropipettes	Eppendorf, Germany
Nutator	
Stereo Microscope	Olympus, USA (SZ61)

3.3. Experiments for Localizing Uzip Expression

3.3.1. Expression Analysis of Uzip

In order to analyse the expression pattern of Uzip; AC783-Gal4 enhancer trap line was crossed with a reproter line that expresses membrane bound GFP under the control of UAS. Besides, Uzip::mCherry Bac line was crossed with flies that have GFP labelled MBs (Figure 3.1).

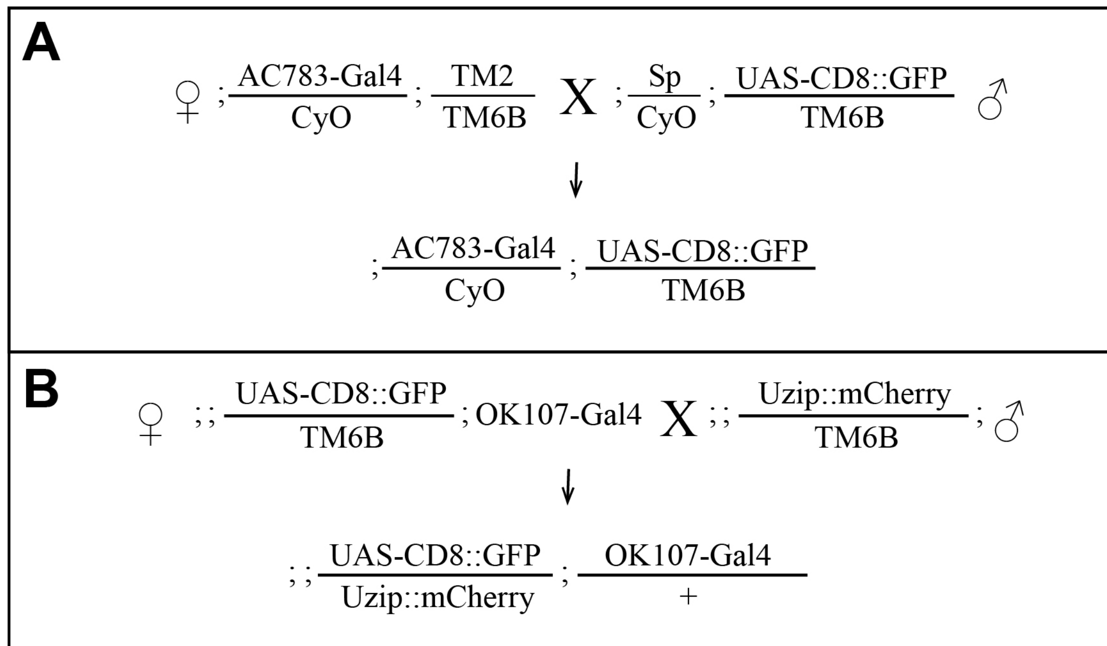

Figure 3.1. Crosses for Uzip localization experiments.

3.3.2. Uzip LOF experiments

LOF analyses were performed by dissection of mutant lines and performing RNAi mediated cell type-specific knockdown experiments. Different Gal4 drivers were combined with UzipRNAi for analysis (Figure 3.2). Similar experiments with different Gal4 driver were performed.

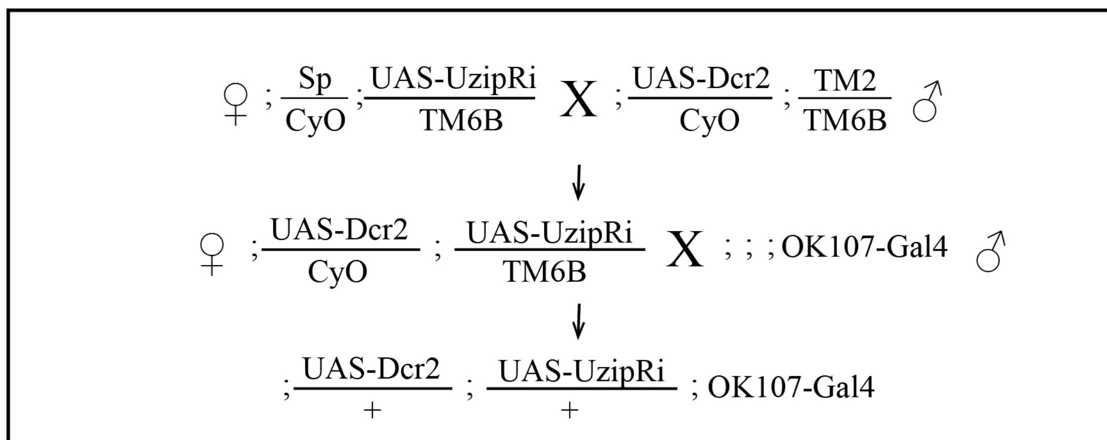

Figure 3.2. Crosses for Kenyon cell specific knockdown experiments.

3.3.3. Molecular Rescue Experiments

In order to mediate cell type-specific molecular rescue experiments Gal4 drivers and UAS-Uzip construct were combined on homoztgous mutant background (Figure 3.4). Similar experiments were performed with different Gal4 driver on different mutant backgrounds.

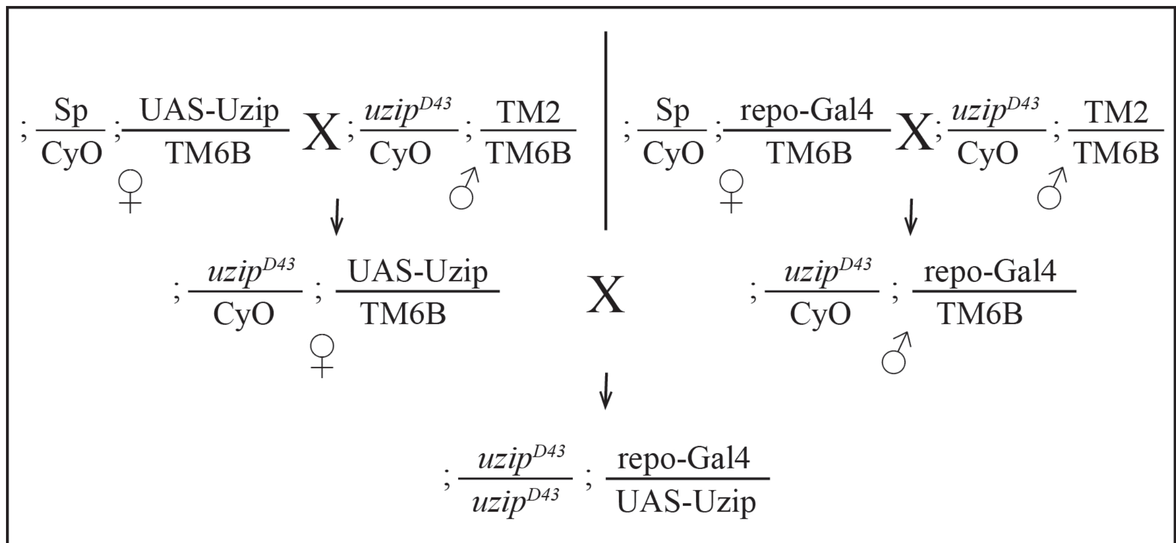

Figure 3.3. Crosses for cell type specific molecular rescue experiments.

3.3.4. Genetic Interaction Analyses

To test genetic interaction, to different mutant lines were crossed and trans-heterozygote progeny was tested for MB phenotypes (Figure 3.4). Similar experiments were performed with different mutant lines.

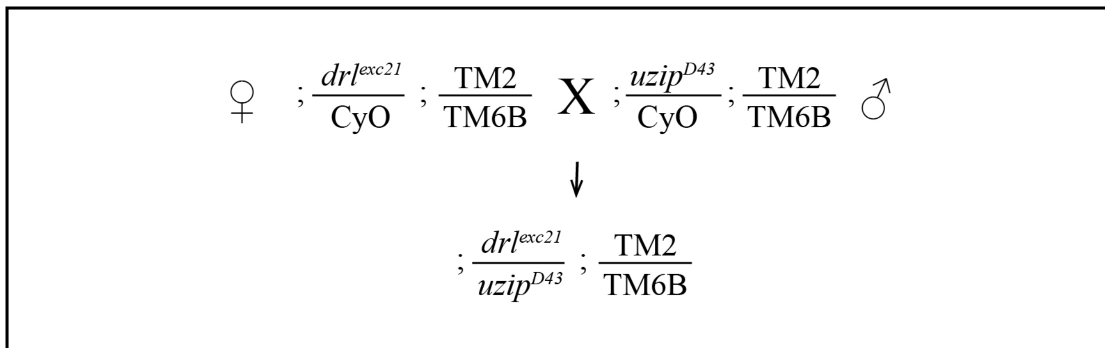

Figure 3.4. Crosses for genetic interaction analyses.

3.4. Histological Techniques

3.4.1. Immunohistochemistry

One to three days old young adults were collected and their brains were dissected. Dissection had been done in one-hour session and the brains were collected in PBS on ice. At the end of each dissection session, antibody stainings were performed on the collected brains.

The fixation of the tissues was done through replacing PBS with PFA solution (4% in PBS) and incubating 20 minutes. Then, the tissues were washed three times with PBX3 for 15 minutes each. Following the tissues were blocked in PAXD solution for 15 minutes and primary antibody solution prepared in PAXD and tissues were incubated in it overnight at 4°C. After that the tissues were washed with PBX3 three times for 15 minutes each. Secondary antibody solution in PAXD prepared and tissues were incubated in it overnight at 4°C. After that the tissues were washed with PBX3 three times for 15 minutes each and mounted on a slide with Vectashield. All the steps were performed at room temperature with 200 rpm agitation unless stated otherwise.

3.4.2. Visualization and Image Processing

Mounted brain tissues were visualized under fluorescent confocal microscope and image processings were done with ImageJ and Adobe Photoshop programmes.

4. RESULTS

Uzip has been identified as a novel cell adhesion molecule by Ding and his collegues, not only because the type and the main function of the molecule was identified for the first time but also it shows no similarities with any of the known CAMs. The same study revealed that Uzip is mainly expressed by glia during embryonic stages of *Drosophila* to mediate axon guidance, and it has been also shown that *CadN* and *wnt5* genetically interact with Uzip (Ding *et al*., 2011).

In previous studies carried out in our lab Selen Zülbahar (2012) showed that olfactory sensory neurons exhibit severe midline crossing defects in *uzip* mutants. In a follow-up study, Kaan Mika (2014) generated a new UAS-Uzip construct and with the use of a functional UAS-UzipRi (TRiP) line, it had been demonstrated that both glial and neuronal expression of *uzip* is required for axon guidance of olfactory sensory neurons. Using a genomic rescue construct in which Uzip was tagged with the mCherry reporter gene to visualize the endogenous localization of Uzip and an enhancer trap line within the Uzip gene (Arzu Öztürk, 2010), Uzip was shown to be expressed by glial cells and some neurons in the adult *Drosophila* olfactory system.

In this study, we aim to identify the role of *uzip* in *Drosophila* mushroom body development. In order to assess this goal, we have analyzed the expression pattern of Uzip in the MB with available transgenic lines. Then functional analyses including loss-of-function, gain-of function and molecular rescue experiments have been performed. Lastly, the genetic interaction partners of *uzip* have been identified.

4.1. Analyses of the Expression Pattern of Unzipped Around Mushroom Bodies

Identification of the expression pattern of a particular gene is an important step of identifying its function. We have made use of three different tools to investigate the expression pattern of Uzip: *uzip* enhancer trap line (Arzu Öztürk, 2010), mCherry-tagged Uzip BAC line (Kaan Mika, 2014), and a α-Uzip antibody (Ece Terzioğlu Kara, 2015). Since the expression pattern of Uzip in the adult brain with each of these tools had been shown in the mentioned studies, we have only focused on the expression of Uzip in the MB neuropil.

The first experiment to localize Uzip experiment was to drive membrane-bound GFP expression with AC783 enhancer trap line. It can be clearly seen that there are Uzip expressing cells in the region of MB neuropil, and co-immunostaining these brains with α-FasII antibody that stains the axons of the α/β and γ neurons, revealed that those Uzip positive cells are not the MB neurons themselves but the cells in close contact with α and β lobes (Figure 4.1 A-A’). These cells have previously been identified as ensheathing glia (Ou *et al*., 2016).

The second line that has been used to investigate Uzip localization is the Uzip::mCherry BAC transgenic line. Uzip::mCherry fusion protein is predicted to be expressed in the same pattern as the endogenous Uzip protein (Kaan Mika, 2014). To reveal the localization of Uzip::mCherry protein around MB neuropil, this BAC transgene had been combined with OK107-Gal4>UAS-CD8::GFP reporter line in which all MB neurons are labelled with membrane-bound GFP. Here as well it can be seen that Uzip::mCherry is expressed widely around MBs with an enrichment around the heel region of the MBs (Figure 4.1 B-B’, arrows).

The last experiment to localize Uzip protein was an immunostaining on wild type *Drosophila* brains with α-Uzip antibody (Ece Terzioğlu Kara, 2015). In these stainings, only the surface of the brain is stained and no staining in the inner layers was observed. As a result, no expression was detected around the MBs (Figure 4.1 C-C’).

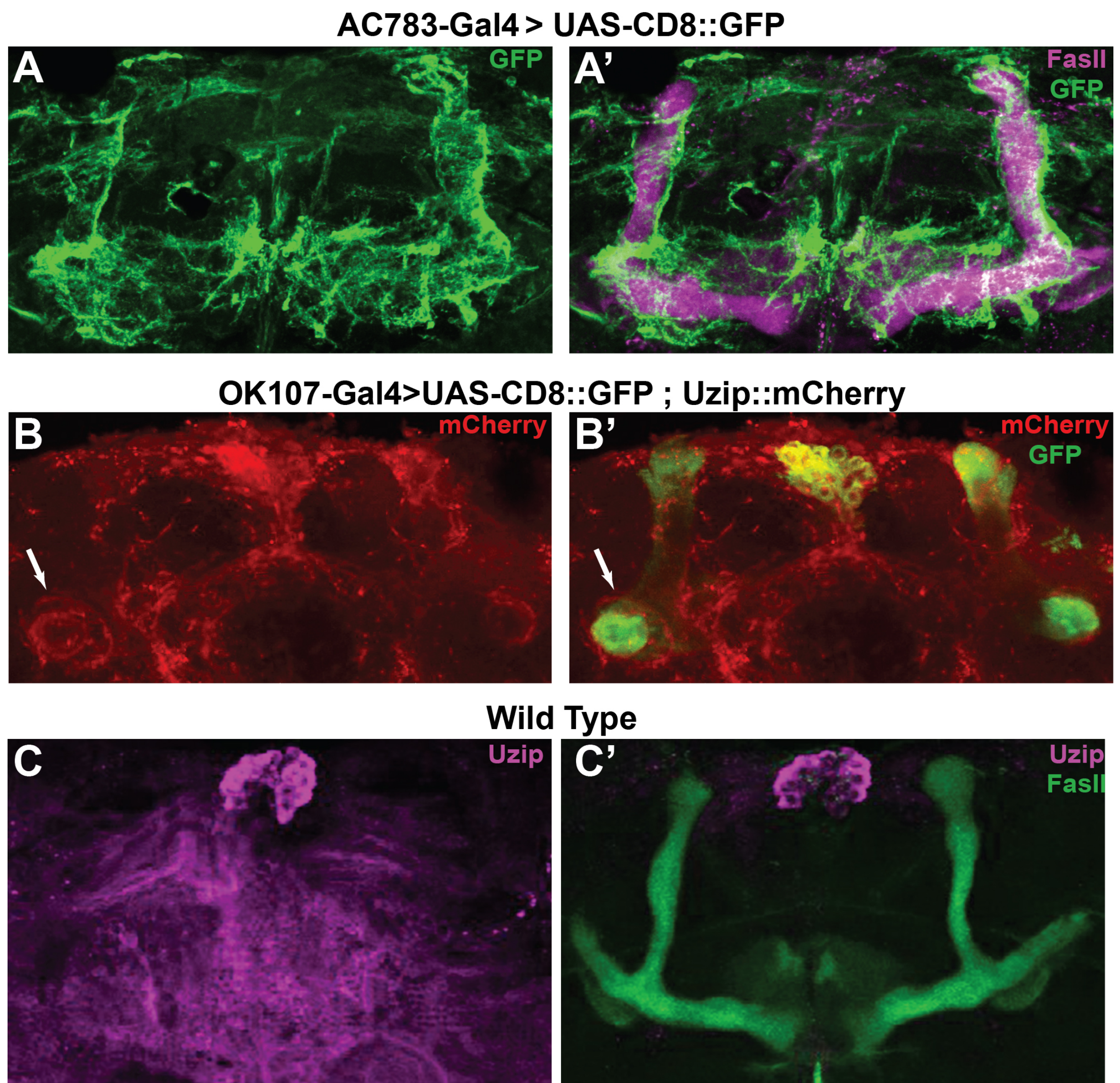

Figure 4.1. Localization of Uzip around mushroom body neuropil. (A) AC783-Gal4 driven membrane bound GFP labels some cell populations around the MB lobes and (A') α, β and γ lobes are labeled with α-FasII antibody staining. (B) Uzip::mCherry fusion protein is also expressed around MB neuropil and (B') labeling membranes of all MB neurons revealed that expression is enriched around heel region (arrowheads). (C) α-Uzip antibody staining around the MB region (C') and only focused on MB neurophil stacks.

4.2. Analyses of Mushroom Body Morphology of *uzip* Mutants

According to the localisation experiments Uzip is mainly expressed by glia associated with the MB at the adult stage. In order to identify whether Uzip has a role in MB development we performed LOF studies. Two mutant alleles of *uzip* are available: $uzip^{D43}$ and $uzip^{23}$, which were generated by Ding and his collegues. The first one is a null allele generated by the deletion of the whole coding sequences of *uzip* with the use of the FLP/FRT system. The latter one is a hypomorphic allele generated via imprecise excision of a PiggyBac transposon located in the 5'UTR of the *uzip* gene (Figure 4.2 A) and shown to express Uzip at lower levels (Ding *et al*., 2011).

The MB morphology of wild type and heterozygote flies for either of the alleles was examined and no morphological differences observed (Figure 4.2 B). However, flies homozygous for the $uzip^{23}$ hypomorphic allele exhibited various developmental MB phenotypes including thin lobes (Figure 4.2 C arrows), and missing and short lobes (Figure 4.2 C' arrowheads). Flies transheterozygous for $uzip^{23}$ and $uzip^{D43}$ were shown to have more severe defects in their MBs. Apart from the phenotypes observed in homozygous hyphomorph mutants, MB axons begin to project on a crooked path (Figure 4.2 D, arrows), and in most severe cases the axons were not able to project anywhere and just grow and crawl around the cell body, generating ball-like structures (Figure 4.2 D', arrowhead). The most severe phenotypes were observed in homozygous null mutants. The neurons that manage to project their axons follow totally random paths but the most abundant phenotype is the generation of ball-like structures, also known as axon crawling (Figure 4.2 E-E').

Immunostaining with the α-FasII antibody not only reveals the MB but also the EB morphology. In transheterozygote animals and null mutants the EB does not form across the midline axis; instead the EB is duplicated and two EBs form on both sides of the midline. These ectopic EBs are malformed in null mutants (Figure 4.2 D, E, E' asterisks).

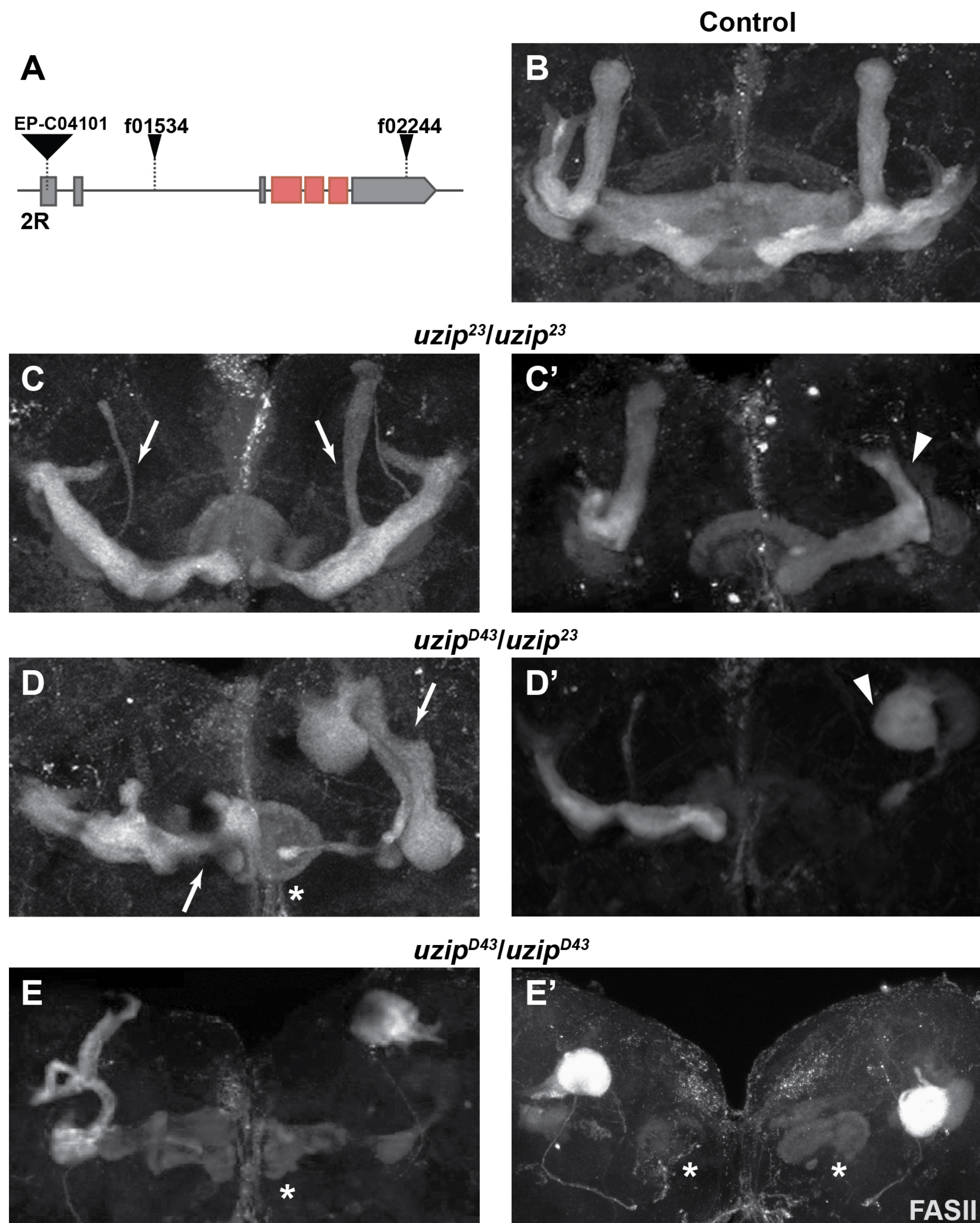

Figure 4.2. Analyses of mushroom body phenotypes in *uzip* mutants. (A) *uzip*^{D43} alele has been generated via deletion of genomic region between f01534 and f02244, and *uzip*^{23} allele has been generated via imprecise excision of piggyBac EP-C04101, which left a 300bp insertion behind. (B) MB morphology of control flies. (C-C') Homozygous hypomorph flies exhibit thin (arrows) and short lobe (arrowhead) phenotypes. (D-D') Transallelic combination of null and hypomorph alleles generates crooked lobes (arrows) and axon stalling (arrowhead) phenotypes.

In addition to the described variance in the severity of phenotypes, the three different mutant backgrounds showed a dramatic variance in the penetrance of phenotypes. In order to understand the dynamics, the observed phenotypes were grouped into three classes according to their severity – axon misguidance, one lobe missing and axon stalling – and the occurence of each phenotype was quantified. The bar graph shows that as Uzip levels decrease in the organism, the severity and occurence of the phenotypes increases (Figure 4.3).

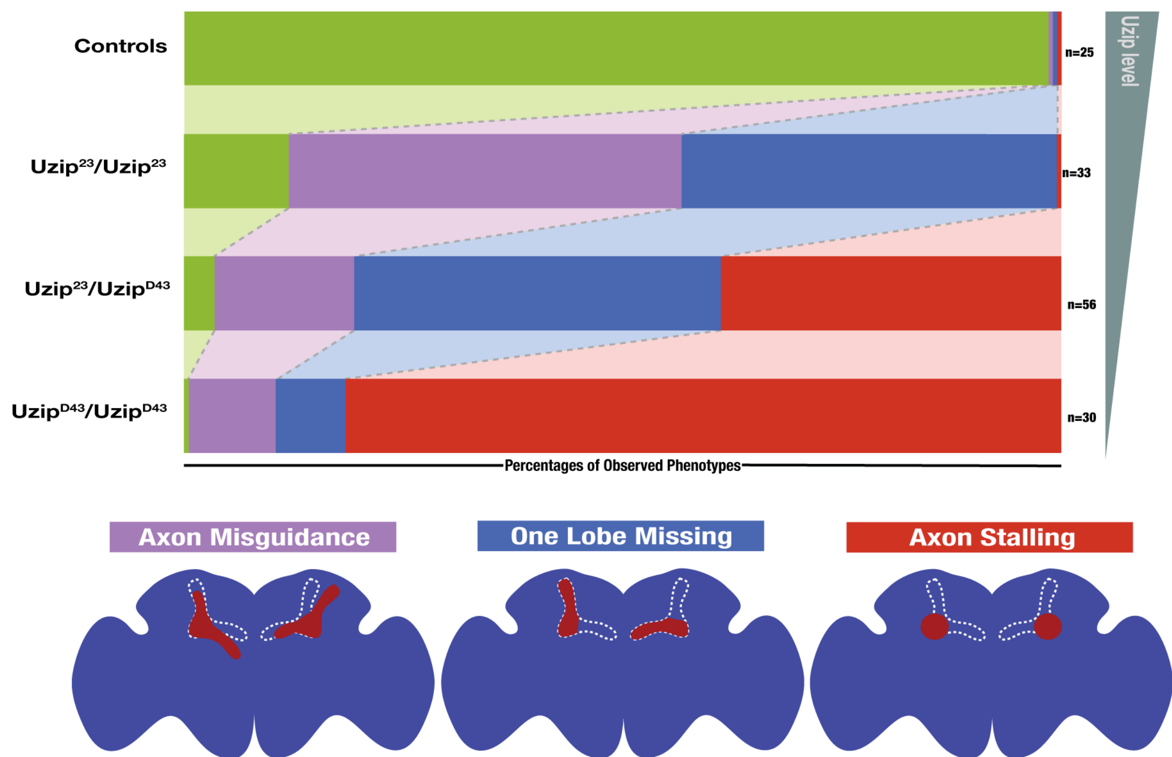

Figure 4.3. Quantification of mushroom body phenotypes in *uzip* mutants. Green bars indicate wild type, purple bars axon misguidance, blue bars MBs with one lobe missing, and red bars MBs with axon crawling phenotypes. The length of each bar is proportional to the percentage of MBs observed with the corresponding phenotype among all investigated MBs of a certain genotype. Two hemispheres of each brain were scored independent of each other and n indicates the number of hemispheres examined. The schemes at the bottom of the graph depict the phenotypes represented by each category.

In order to address the temporal identity of the phenotypes, null mutant MBs at 3rd instar larval stage were analysed. At this stage, α'/β' lobes are generated, but their contribution to the neuropil structure is quite small, so the MB structure mainly consists of γ neurons. α-FasII staining revealed that MBs of homozygous null mutant flies are indistinguishable from those of wild type control flies (Figure 4.4). This data shows that Uzip is not required for early γ neuron development.

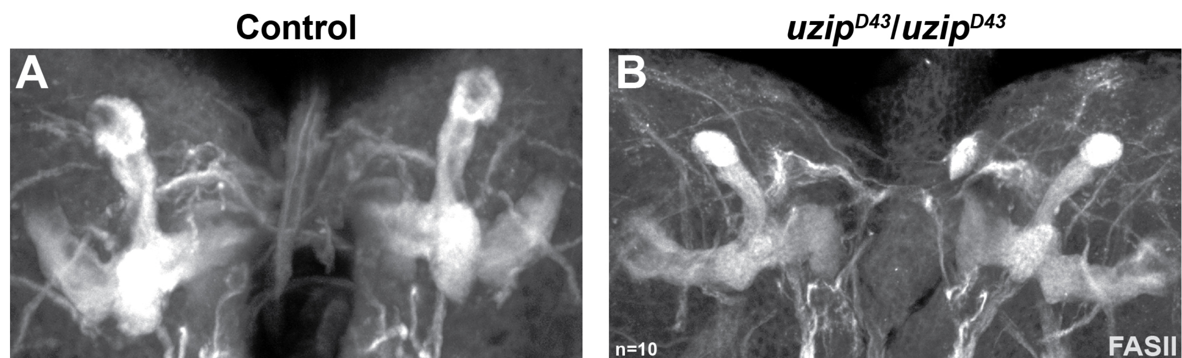

Figure 4.4. Analyses of MBs of *uzip* mutants at late larval stage.
(A) The MBs of wild type larval animals revealed by α-FasII staining are indistinguishable from (B) the MBs of *uzip* null mutants. n is the number of hemispheres examined.

4.3. Analyses of the Effect of Neuronal Knock-Down of Uzip on Mushroom Body Development

Analysis of *uzip* mutant flies revealed that *uzip* has a crucial role in MB neuropil development. In order to understand in which cells uzip function is required, we designed experiments to silence *uzip* expression in certain cell populations. Since MBs consist only of neurons, initial experiments were designed to investigate the role of Uzip in MB development by downregulating its function in neurons.

Silencing Uzip expression in all CNS neurons with the pan-neuronal driver *elav*-Gal4 results in one lobe missing phenotype (Figure 4.5 B). This phenotype has previously been observed in *uzip* mutants as well. Thus, it can be concluded that pan-neuronal knockdown of Uzip phenocopies the mutant phenotype. After confirming that Uzip function in neurons is crucial for proper MB development, more specific neuronal drivers

were tested. In the next set of experiments a Kenyon cell-specific driver, *OK107-Gal4*, was used. Downregulation of Uzip only in MB neurons using this driver, lobe missing and axon misguidance phenotypes were observed (Figure 4.5 C-C'). Subsequently, to enhance the silencing effect *UAS-Dicer2* was expressed along with *UAS-UzipRi* and in this genotype all mutant phenotypes were recapitulated (Figure 4.5 D-D').

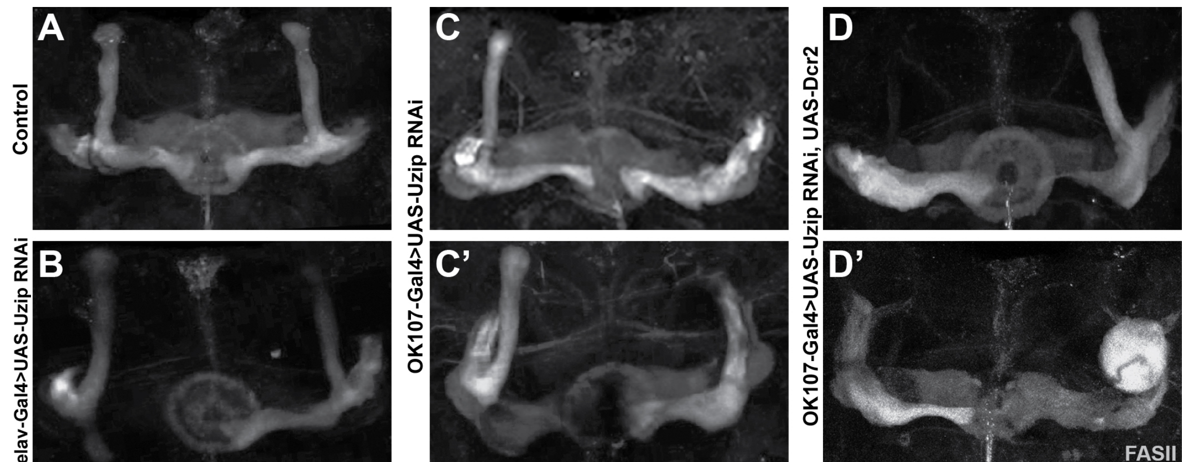

Figure 4.5. Analyses the phenotypes caused by neuronal Uzip knockdown. When adult MB structures are examined through α-FasII staining, it had been observed that (A) all four different control lines (*elav*-Gal4 / *OK107*-Gal4 / UAS-*UzipRi* / *OK107*-Gal4>UAS-*Dcr2*) have normal MBs wheres (B) pan-neuronal *uzip* knockdown and (C-C') silencing of *uzip* only in MB neurons results in axon guidance defects. (D-D') Enhancing the effeciency of RNAi via overexpressing Dcr2 enhances the severity of the phenotypes.

In the previous mutant analysis experiments it was shown that as Uzip levels decrease in the organism penetrance and severity of the MB phenotypes increase. In order to test wheher this phenomenon is valid for neuronal Uzip as well, the severity and occurence of the phenotypes were quantified. When the efficiency of the RNAi effect was boosted via overexpression of Dcr2, decreasing the Uzip levels in Kenyon cells further, the severity and occurence of the phenotypes increased. While more than half of the MBs investigated appeared WT and no axon stalling phenotypes were observed in *OK107-Gal4>UAS-UzipRi* flies, no WT MBs was observed in *OK107-Gal4>UAS-UzipRi, UAS-Dcr2* background and the occurence of all three classes of phenotypes was increased (Figure 4.6).

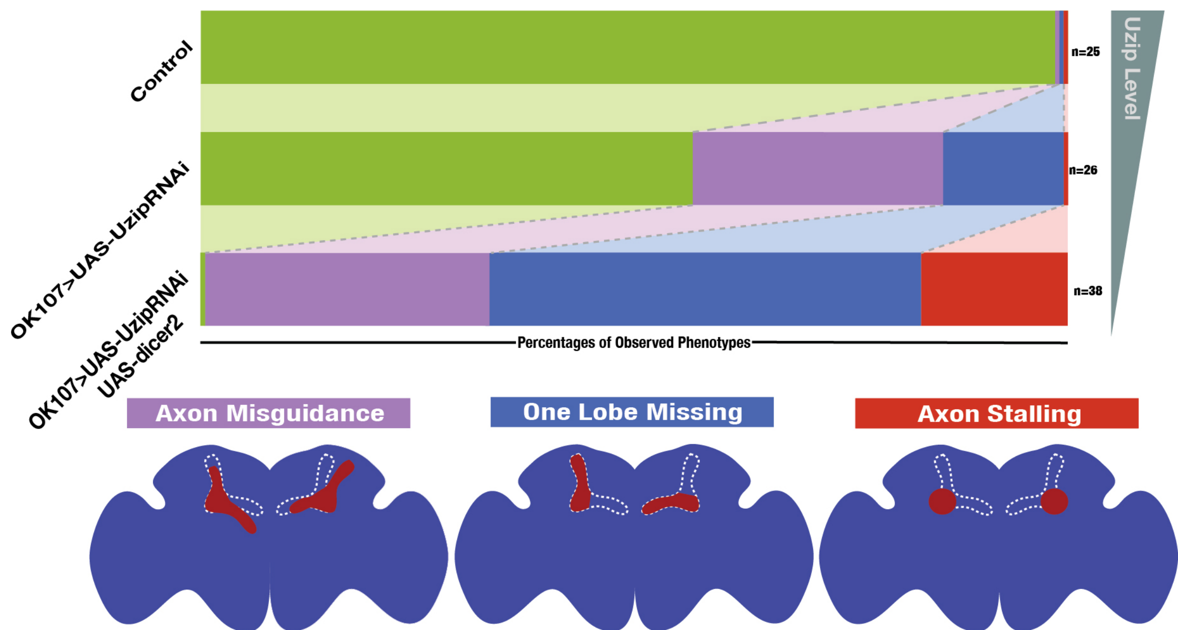

Figure 4.6. Quantification of MB phenotypes in MB specific knockdown experiments. Green bars indicate wild type, purple bars axon misguidance, blue bars MBs with one lobe missing, and red bars MBs with axon crawling phenotypes. The length of each bar is proportional to the percentage of MBs observed with the corresponding phenotype among all investigated MBs of a certain genotype. Two hemispheres of each brain are scored independent of each other and n indicates the number of hemispheres examined. The schemes at the bottom of the graph depict the phenotypes represented by each category.

4.3.1. Testing the Specifity and Efficiency of Different UzipRNAi Lines

Gene silencing experiments with RNAi lines always hold the risk of off-target effects. The siRNA construct designed to target a certain mRNA may affect the expression of some other genes (Seinen *et al*., 2011). And in such a situation, the observed phenotype could arise from or enhanced by these off-targets.

Even though knockdown experiments with the *UzipRi* construct phenocopies the mutant phenotypes, confirming the specifity of this RNAi construct is still crucial. In order to do that, different RNAi constructs targeting different regions of Uzip mRNA were used.

There are three main RNAi libraries, namely GD, KK and TRiP, publicly available in two different *Drosophila* resource centers. None of the libraries cover the whole *Drosophila* genes but luckily, there is one RNAi construct in each of the libraries. All of the lines were obtained and crossed with *OK107-Gal4>UAS-Dcr2* flies. The MB phenotypes of the progeny were analyzed and scored. The result of this experiment revealed that all of the three RNAi lines are leading to MB phenotypes observed in *uzip* mutants. GD construct leads to lobe missing and axon misguidance (Figure 4.7 A-A'), the KK leads to the same phenotypes and additionaly to axon stalling (Figure 4.7 B-B') and the TRiP line also displays all the three types of phenotypes (Figure 4.7 C-C'). Since each of this constructs was designed to target a different region of *uzip* mRNA, it is very unlikely for all of them to have the same off-targets. Thus, these data confirm that the phenotypes observed with RNAi silencing experiments result from decreased levels of Uzip.

When the penetrance of the different phenotypes were quantified and compared with each other, the TRiP line-mediated knockdown seemed to result in more severe phenotypes with a higher penetrance (Figure 4.7 D) as compared to the other RNAi lines. Since the TRiP line mediates the most efficient knockdown, it was used for all RNAi-based knockdown experiments in this study.

4.3.2. Slincing Uzip Expression in Mushroom Body Neuron Subtypes

Previous LOF experiments proved that Uzip expression in neurons, specifically in Kenyon cells, is required for proper MB development. There are three types of MB neurons, namely α/β, α'/β' and γ. It has been known that those neurons are born in a sequential manner and early born neurons guide later born ones to their correct location in the brain. Especially α'/β' neurons were demonstrated to be guiding α/β neurons (Goossens *et al*., 2011; Siegenthaler *et al*., 2015).

Visualizing MB morphology with immunostaining using a α-FasII antibody reveals only α, β and γ lobes. In order to observe the morphology of α', β' lobes, *OK107-Gal4* construct was combined with *UAS-CD8::GFP* along with *UAS-Dcr2* and *UAS-UzipRi*. In

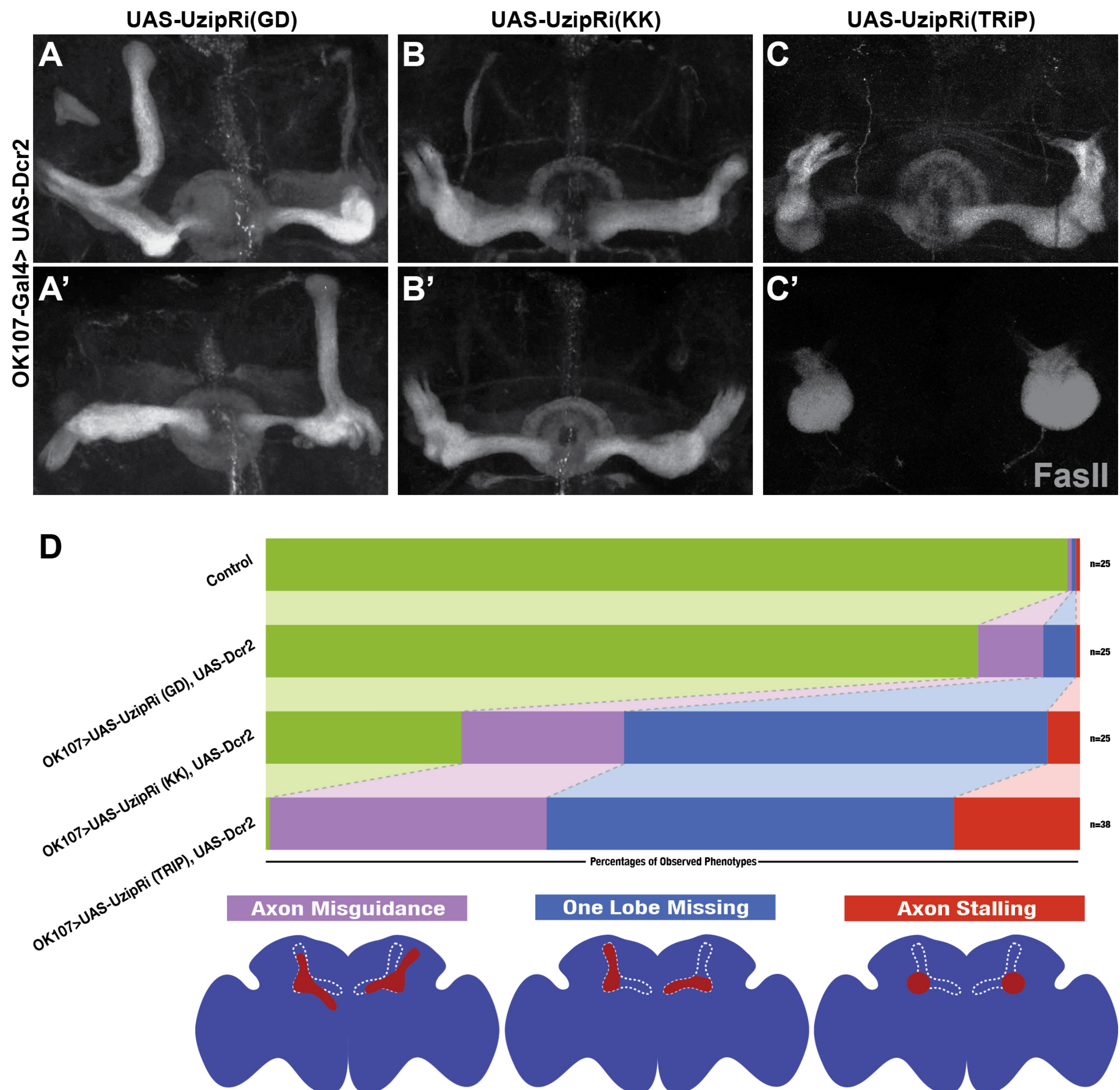

Figure 4.7. Assesing the specifcty and efficiency of UzipRi lines.
Silencing in MB neurons with (A-A') GD, (B-B') KK and (C-C') TRiP lines yield the same set of phenotypes but (D) with different penetrance. Green bars indicate wild type, purple bars axon misguidance, blue bars MBs with one lobe missing, and red bars MBs with axon crawling phenotypes. The length of each bar is proportional to the percentage of MBs observed with the corresponding phenotype among all investigated MBs of a certain genotype. Two hemispheres of each brain are scored independently from each other and n indicates the number of hemispheres examined. The schemes at the bottom of the graph depict the phenotypes represented by each category.

those flies all MB neurons are labelled with membrane-bound GFP. Immunostaining of brains of this genotype with α-FasII antibody will label all later born neuron subtypes. Thus, early and late-born neurons can be visualized and distinguished from each other in this was as α/β neurons will be positive for GFP and α-FasII staining, whereas α'/β' neurons will only be positive for GFP. In one of the MBs of these flies it was observed that the α lobe is missing while the α' lobe was formed normally. The other MB of the same brain shows that both α/β and α'/β' neurons follow the same fate and display an axon stalling phenotype (Figure 4.8 A).

In order to test whether Uzip is required for neuron-neuron interactions between different types of MB neurons, neuron subtype-specific knockdown experiments were performed with different Gal4 lines. First, the γ neuron-specific line *R16A06-Gal4* was used to drive *UAS-UzipRi* and neither γ nor α and β lobe morphologies were disturbed (Figure 4.8 B). When *uzip* expression was silenced specifically in α'/β' neurons with *c305-Gal4*, an α lobe missing phenotype was observed even though *uzip* expression in α/β neurons was not disturbed (Figure 4.8 C). A one lobe missing phenotype was observed again when the knockdown was done only in α/β neurons (Figure 4.8 D).

Taken together, this data suggests that Uzip is required for early born α'/β' neurons to guide later born α/β neurons. And Uzip expression from both of the neuron populations is required. In order to test whether this role is only valid for α/β pioneer neurons we knockdown Uzip only in those cells with *c708-Gal4* driver, but the results demonstrated that Uzip is not required to be expressed by α/βp neurons for other neurons to follow them (Figure 4.9).

4.4. Analyses of the Effect of Neuronal Knock-Down of Uzip on Ellipsoid Body Development

Even though it has been known for a long time that EB is an important part of the *Drosophila* central brain complex, the knowledge about its function and development is very limited. During the investigation of the role of *uzip* in MB development, it was

unintentionally revealed that *uzip* is also important for EB development. In order to get some insight about the role of *uzip* in EB development, LOF experiments were performed.

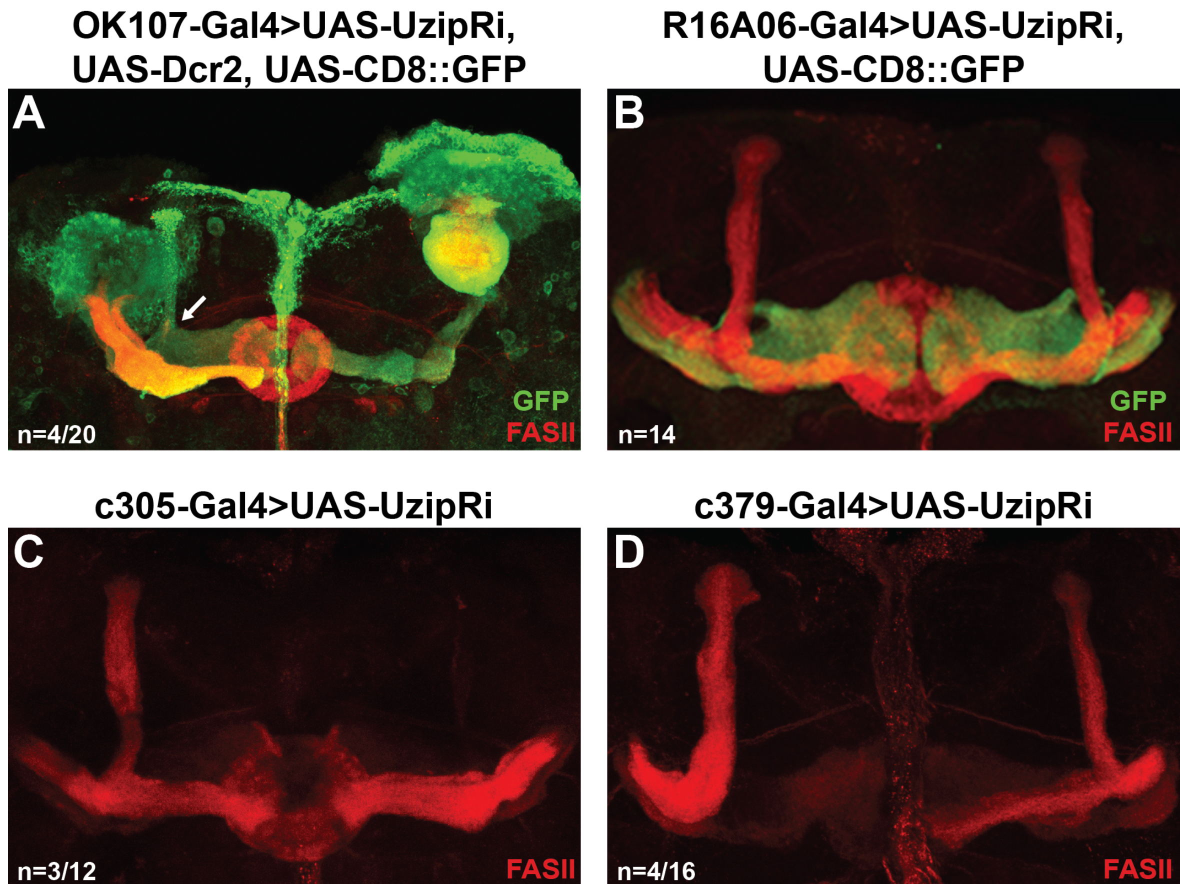

Figure 4.8. MB neuron subtype specific knockdown experiments. (A) All MB knockdown results in α lobe missing and axon stalling phenotypes. In the left hemisphere, α' lobe (arrow) is formed normally whereas the α lobe is missing. (B) Silencing *uzip* only in γ neurons doesn't result in any MB defects but silencing only in (C) α'/β' and (D) α/β neurons both result in lobe missing phenotypes. n indicates number of hemispheres examined.

First the knockdown lines generated for MB experiment had been used to identify EB phenotypes. Immunostaining with α-FasII antibody revealed that none of the control lines – *elav-Gal4*, *OK107-Gal4* and *UAS-UzipRi* – display any perturbations in adult EB morphology. However, when *uzip* expression was silenced pan-neuronally two EBs instead of one was formed and the structures did not have the firm elliptic shape of the WT ones.

On the other hand, Kenyon cell-specific knockdown experiements with the *OK107-Gal4* line did not result in any EB phenotypes (Figure 4.10).

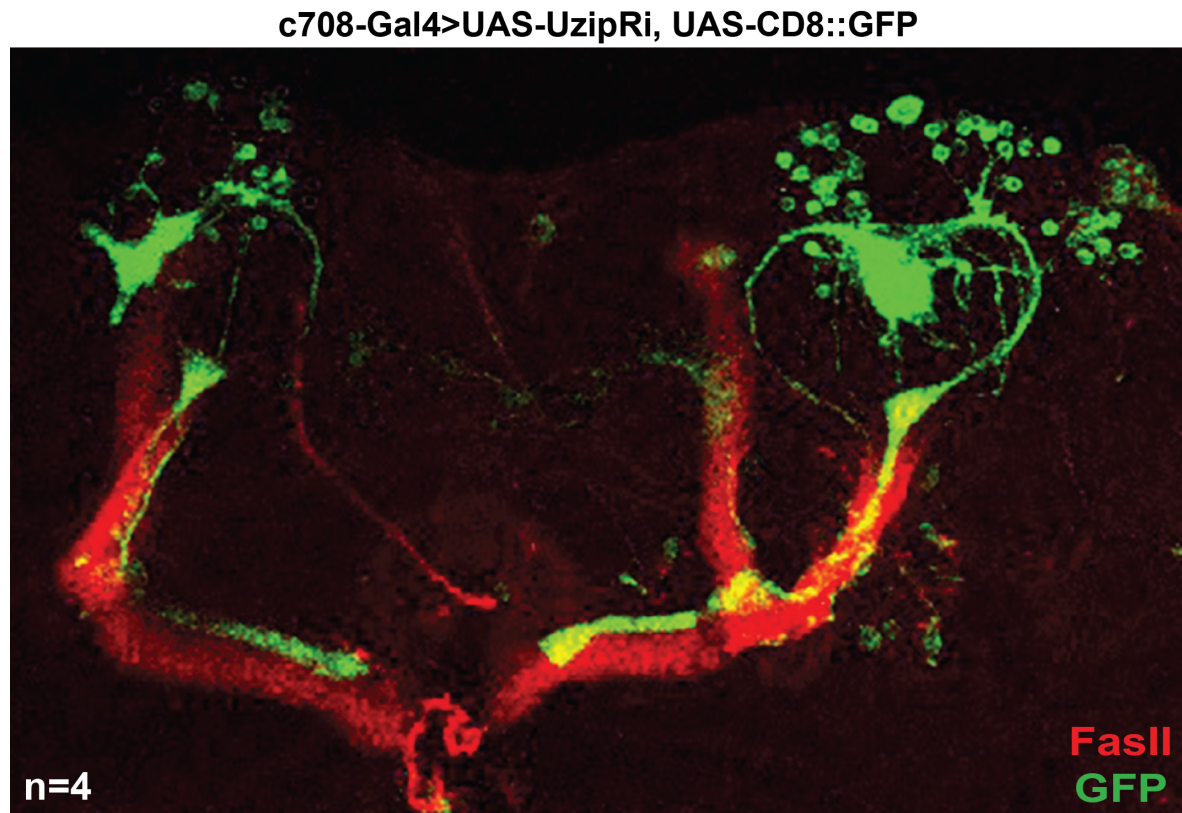

Figure 4.9. Uzip knockdown in α/β pioneer neurons. Silencing of *uzip* expression in α/β pioneer neurons neither results in a morphological phenotype in these neurons nor the rest of the α and β lobes. n indicates the number of brains examined.

After confirming that EB phenotypes observed in mutant lines result from neuronal loss of Uzip, EB-specific *R15F02-Gal4* line had been used to silence Uzip function only in EB neurons. Membrane-tagged GFP expression under the control of *R15F02-Gal4* control was used to visualise EB structure and the EB morphology of knockdown lines had no difference than the control ones (Figure 4.11).

4.5. Analyses of the Effect of Glial Knock-Down of Uzip on Mushroom Body Development

Previous experiments focusing on neuronal requirement of Uzip, had demonstrated that Uzip is required for guidance of α/β neurons by α'/β' neurons. While this model for Uzip function explains the phenotypes observed in α and β lobes it does not explain the phenotypes observed in α'/β' neurons. Consistent with a previous study where Uzip has shown to be expressed in glial cells (Ding *et al*., 2011), Uzip expression in the brain was shown to be mainly glial as well. Thus, we speculated that glial Uzip might mediate MB development. In order to test this hyphothesis, LOF studies targeting glial Uzip were performed.

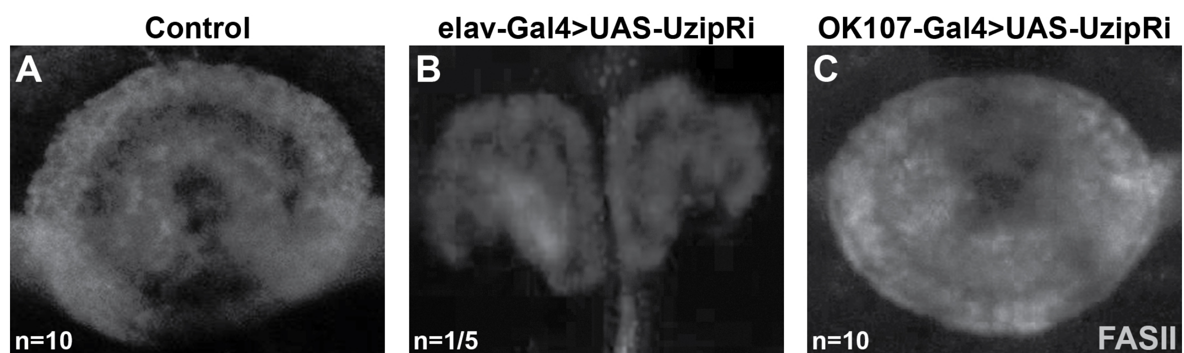

Figure 4.10. Effect of Uzip knockdown on EB morphology. (A) Neither of the control lines (*elav-Gal4 / OK107-Gal4 / UAS-UzipRi*) show anomalies of the EB structure. (B) When *Uzip-Ri* is expressed with pan-neuronal *elav-Gal4* two abnormal EB are formed whereas (C) expression of *Uzip-Ri* in Kenyon cells do not lead to any EB phenotype. n is the number of brains examined.

4.5.1. Pan-Glial Uzip Loss-of-Function

In order to manipulate the *uzip* expression in glial cells the pan-glial driver *repo-Gal4* was used. This enhancer-trap line drives Gal4 expression in all glial cells except the midline glia (Sepp *et al*., 2001). When *uzip* expression was silenced in glial cells with pan-glial *repo-Gal4* driver and *UAS-UzipRi* the resulting MBs were indistinguishable from control flies (Figure 4.12 A-B).

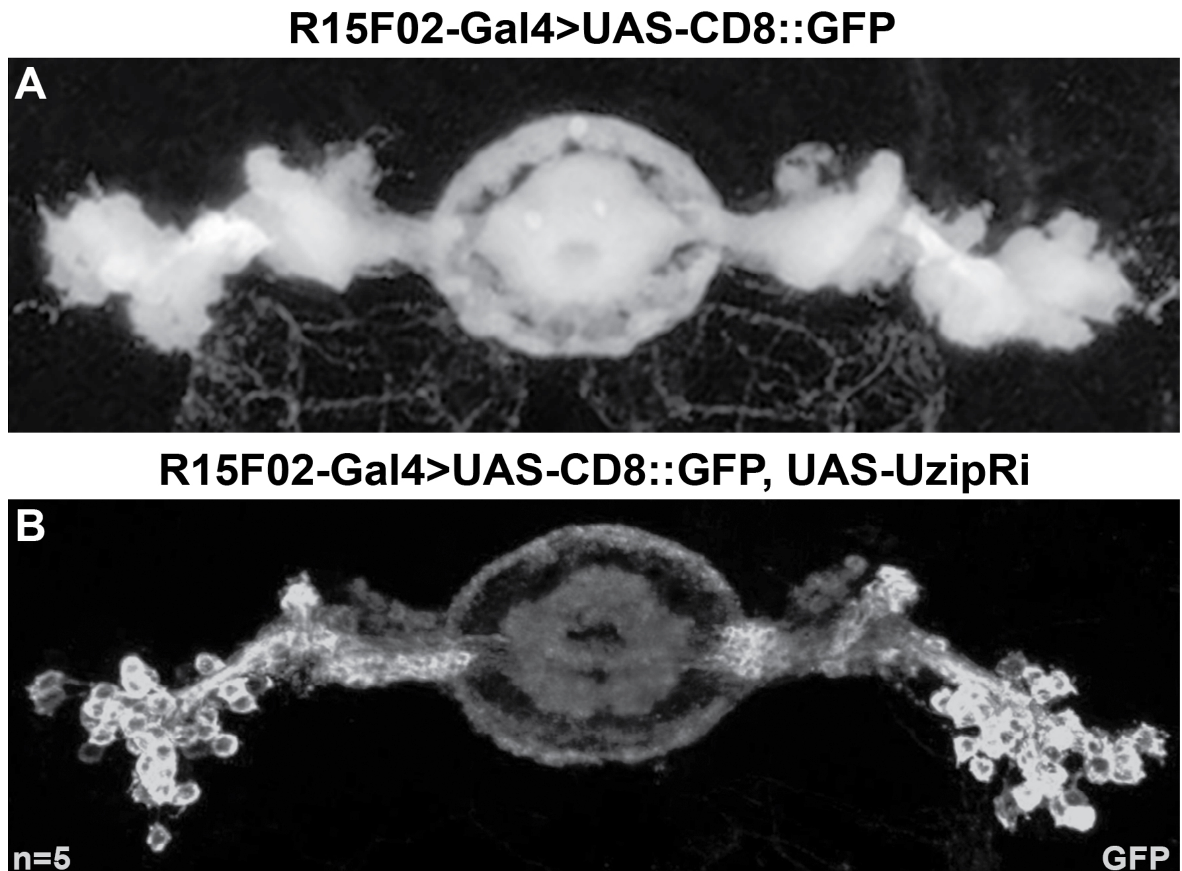

Figure 4.11. Analyses of EB specific knockdown. (A) The EB structure of control animals is identical with the structure of the EB in (B) knockdown lines. n indicates the number of brains examined.

To exclude the possibility that glial knockdown with one copy of *repo-Gal4* was not sufficient a second copy of *repo-Gal4* on a different chromosome was used. This second *repo-Gal4* driver line, *repo4.3-Gal4*, was generated by the group of Prof. Christian Klämbt (Univ. Münster) and kindly shared with us. Interestingly, enhancing the expression of *repo-Gal4* and ultimately the knockdown effect resulted in axon misguidance and lobe missing phenotypes (Figure 4.12 C).

As a complemantary approach, the RNAi pathway was enhanced further by over-expression of Dcr2 protein along with the *UzipRi* construct under the control of *repo-Gal4*. This experiment resulted in a previously not observed phenotype, β lobe fusion (Figure 4.12 D).

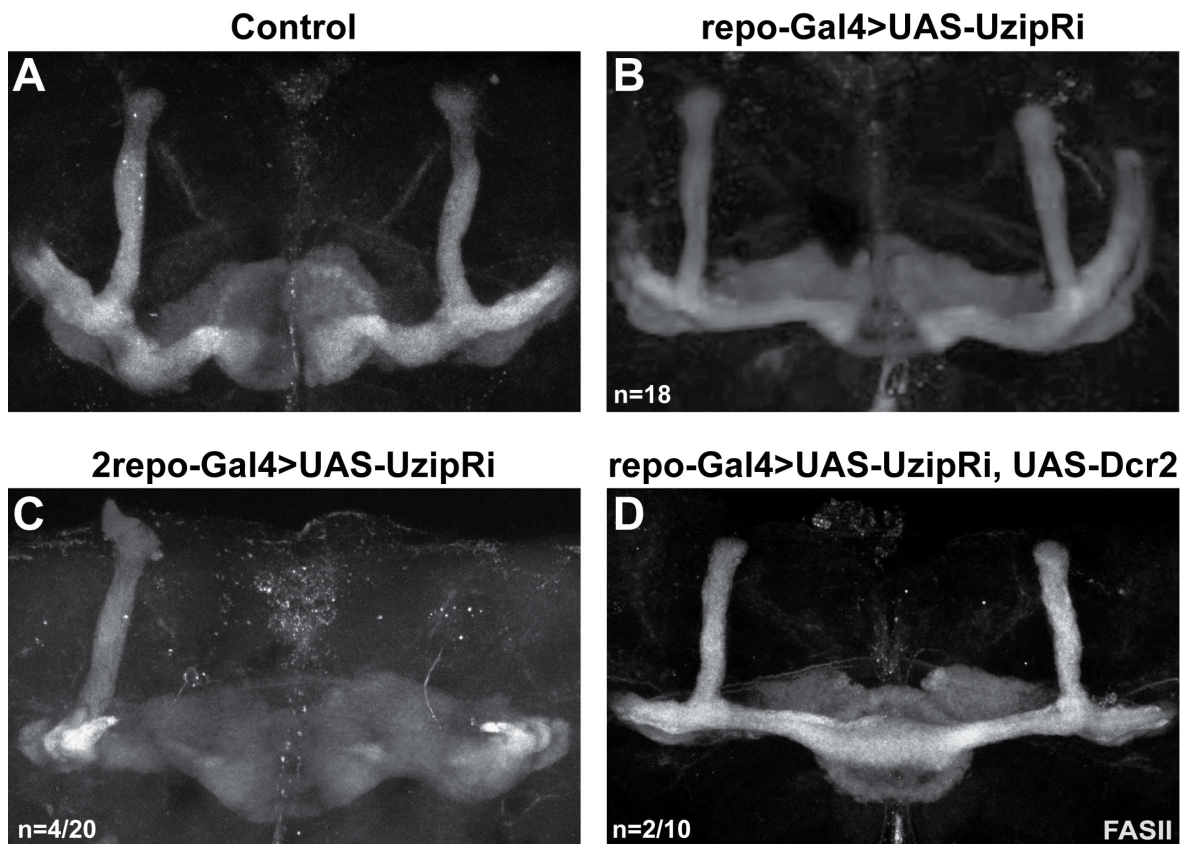

Figure 4.12. Analyses of glial Uzip loss-of-function experiements. (A) Control flies (*repo-Gal4* / *2repo-Gal4* / *repo-Gal4, UAS-Dcr2* / *UAS-UzipRi*) and knockdown with single *repo-Gal4* driver individuals has morphologically normal MBs. (C) Using two *repo-Gal4* constructs results in α lobe overextension phenotype on the left MB and α lobe missing phenotype in the right one. (D) Overexpressing Dcr2 in single repo driver knockdown background results in β lobe fusion phenotype. n indicates the number of hemispheres for B and C, and number of brains for D.

4.5.2. Analyses of the Role of Uzip in TIFR – MB Interactions

The only glial structure that was previously shown to be important for MB axon guidance is Transient Interhemispheric Fibrous Ring. This transient structure is formed by four glial cells and mediates the position of the β lobe tips with respect to the midline. When this structure is ablated, the lobular structure of MBs collapses and all neurons cross the midline and merge with the MB neurons of the contralateral side (Simon *et al.*, 1998). In our lab, it was shown that the TIFR structure is disrupted in *uzip* null mutants, and

appears to be shortened on the ventral side; however, this disruption did not interfere with the midline crossing of olfactory sensory neurons (Kaan Mika, 2014). Here, we investigated a possible role of Uzip in MB development.

Uzip was downregulated specifically in TIFR glia using the *C442-Gal4* line known to specifically label TIFR glia (Simon *et al*., 1998). Downregulation of Uzip resulted in fusion of β lobes, whereas no phenotype was observed in control brains (Figure 4.13 A-B). This phenotype resembles the previously described TIFR ablation phenotypes, although if appears somewhat milder. Uzip levels were further decreased by overexpression of *Dcr2* along with *UzipRi*, in addition to a β lobe fusion phenotype the α lobe collapsed onto the midline. This phenotype appears to fully phenocopy the TIFR ablation phenotypes (Figure 4.13 C). Additionally, some overexpression experiments were performed in the framework of this study. Overexpression of Uzip using the TIFR glia-specific driver *C442-Gal4* and a *UAS-Uzip* construct generated in our lab (Kaan Mika, 2014), resulted in more neurons collapsing on the midline (Figure 4.13 D). These experiments show that Uzip expression by TIFR glia is required for proper MB development.

4.5.3. Assesing the Importance of Uzip Expression by Ensheathing and Astrocyte-like Glia on Mushroom Body Development

Previous experiments showed that Uzip expression by glia is required for MB development. The only glial subtype known to regulate axon guidance of MB neurons, TIFR glia, is expressing Uzip to regulate MB development. The next question was if other glial subtypes are regulating MB axon guidance by expressing Uzip.

Two main glial subtypes are known to be tightly associated with the neuropils in the *Drosophila* CNS. The first subtype comprises astrocyte-like glia that is in close contact with MB neurons and important for γ neuron remodelling during pupal stages. The localisation of the cells and their direct association with Kenyon cells at the molecular level raised the question whether astrocyte-like glia could be important for MB axon guidance? In order to answer this question in the context of Uzip research, Uzip was knocked down specifically in astrocyte-like glia usingthe astrocyte glia-specific driver

Alrm-Gal4. However, no phenotype was observed in this genetic background indicating that Uzip function is not required in astrocyte-like glia for MB development (Figure 4.14 A-B).

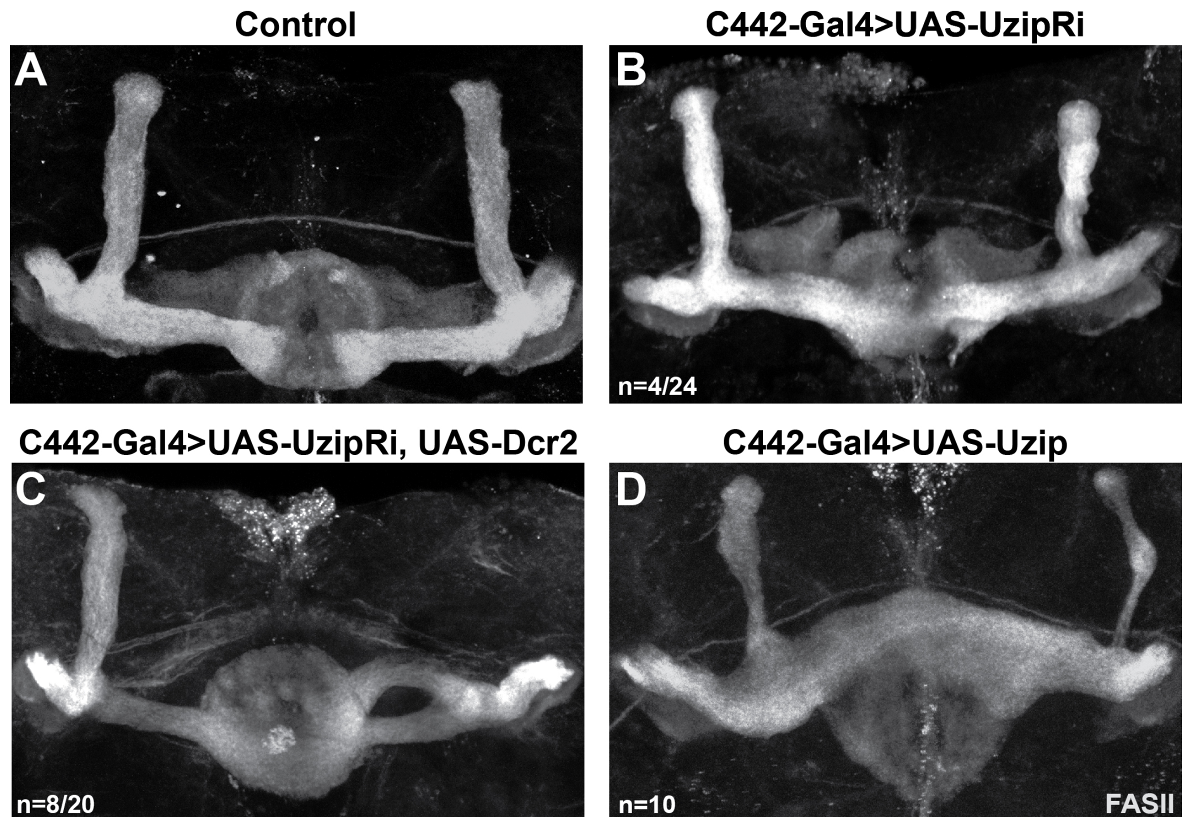

Figure 4.13. The role of Uzip on TIFR-MB interactions. All control lines (*C442-Gal4* / *C442-Gal4, UAS-Dcr2* / *UAS-UzipRi*) have morphologically normal MBs whereas (B) Uzip knockdown in TIFR glia results in β lobe fusion phenotype (C) and collapse of lobes on midline when combined with Dcr2 overexpression. (D) Uzip overexpression in TIFR glia also results in MB neuron collapse on midline. n represents the number of brains examined.

The second glial subtype known to be in close contact with developing and adult MB neurons are ensheating glia. These glia are ensheating all major neuropils in the CNS and are known to act as phagocytes after axonomy (Doherty *et al*., 2009) and important for regulation of different behaviors (Comas *et al*., 2004; Seugnet *et al*., 2011). Downregulation of Uzip expression using the ensheating glia-specific *NP6520-Gal4* driver did not cause any morphological phenotypes in the MB (Figure 4.14 C).

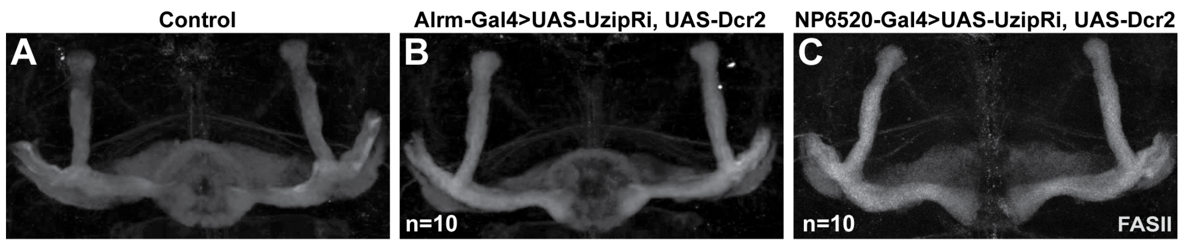

Figure 4.14. Analyses of Uzip requirement by MB associated glia. (A) All control lines (*Alrm-Gal4, UAS-Dcr2* / *NP6520-Gal4, UAS-Dcr2* / *UAS-UzipRi*) have wild type MBs. Downregulation of Uzip in neither (B) astrocyte-like glia nor (C) ensheating glia results in any morphological MB defects. n represents the number of hemispheres examined.

Taken together these results indicate that Uzip does not seem to function in the two major glial subtypes associated closely with the mushroom body cells or neuropil, although it appears to be localized to the latter one. However, Uzip appears to function in TIFR glia to regulate MB development.

4.6. Molecular Rescue Experiments

The results obtained in this study indicate that Uzip function in subsets of neurons and glia are required for MB development. Loss-of-function studies showed that at least one neuronal sub-population, Kenyon cells, and at least one glial cell population, TIFR glia, regulate MB development by expressing Uzip.

To further investigate if Uzip function is sufficient in different cell types, molecular rescue experiments were performed in *uzip* mutant background by re-expressing *uzip* with cell type-specific drivers. MB phenotypes were analysed by immunostaining with α-FasII antibody and the level of rescue was assessed.

4.6.1. Rescue with Uzip BAC Transgenic Lines

Uzip mutants exhibit severe MB phenotypes as shown in section 4.2. These mutants were generated via manipulating the genomic locus of *uzip*. While the expectation is that only the *uzip* locus was manipulated, every genomic perturbation bears the risk that other

parts of the genome are affected, which could contribute to the observed phenotype. In order to confirm the observed MB phenotypes of *uzip* mutants only result from loss of *uzip* expression, rescue experiments with a genomic rescue contructs, Uzip and Uzip::mCherry, were performed. These constructs was generated using a BAC construct that comprises the whole genomic locus of Uzip including all putative upstream and downstream regulatory sequences (Kaan Mika, 2014).

When this constructs were introduced to the homozygous mutant *uzip*^{D43} null allele, MB morphology was fully rescued and reverted from an axon stalling phenotype to the wild type (Figure 4.15). This confirms that the phenotypes observed in *uzip* mutants result from loss of Uzip, as introducing Uzip back to the cells reverses the phenotypes back to the wild type state. Later, Uzip::mCherry transgene was also used for Uzip localization studies as shown in section 4.1.

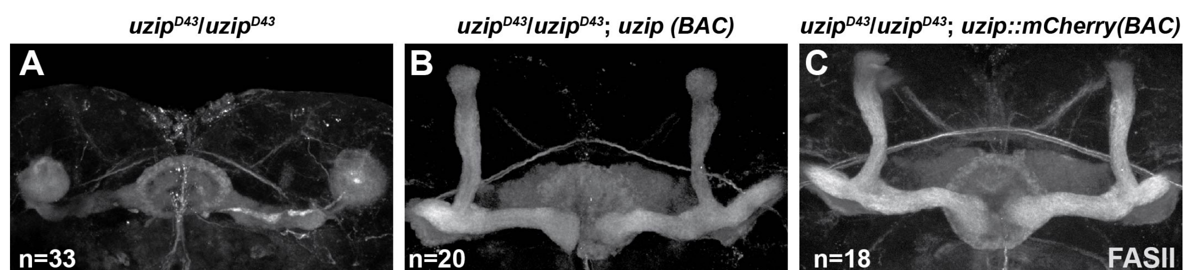

Figure 4.15. Molecular rescue with *Uzip* BAC constructs.
(A) *uzip* null mutants exhibit axon stalling phenotypes and (B) introducing *uzip* transgene or (C) *uzip::mCherry BAC* transgene to the mutants reverses the MBs to the wild type state. n indicates the number of hemispheres examined.

4.6.2. Neuron-Specific Molecular Rescue Experiments

As shown in section 4.3, Uzip expression in neurons is required for MB development. In order to see whether the neuronal Uzip expression is sufficient for normal MB projection, neuron-specific molecular rescue experiments were performed.

Uzip was misexpressed with a transgenic *UAS-Uzip* construct that was generated in our lab (Kaan Mika, 2014). Initially, neuronal rescue experiments using the pan-neuronal

driver *elav-Gal4* were attempted, however none of the several independent crosses gave the desired progeny. Then the strategy was changed and an indirect rescue experiment was performed. Here, a ubiquitous driver *da-Gal4* that drives expression in neurons and glia was used and glial expression was repressed by introducing a repo-Gal80 construct allowing Uzip overexpression only in CNS neurons. The neuronal Uzip expression in this background was sufficient to rescue the axon guidance phenotypes drastically (Figure 4.16). However, the rescued MBs did not resemble the WT ones completely as still some axon guidance phenotypes like ectopic lobe formation could be observed, although with drastically decreased penetrance and severity as compared to full mutants.

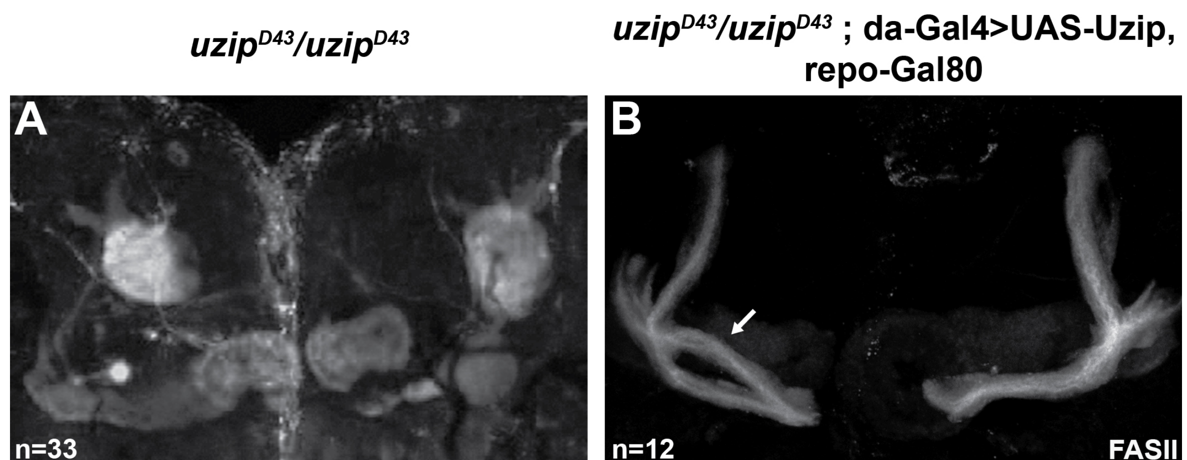

Figure 4.16. Rescue of neuronal Uzip in the null mutant flies.
(A) The null mutants exhibits axon stalling phenotypes whereas (B) only neuronal expression of Uzip rescues MB axon guidance phenotypes significantly, although some phenotypes such as ectopic lobe formation can be observed (arrow).

Rescue experiments using a Kenyon cell-specific driver was attempted to assess the cell-autonomous function of Uzip in MB neurons. However, the attemps to prove this point was inconclusive since the Kenyon cell specific OK107-Gal4 driver is located on the 4th chromosome, and combination of the driver along with UAS-Uzip construct in *uzip*^{D43} null mutant background was not possible in our hands.

4.6.3. Glia-Specific Molecular Rescue Experiments

Cell type-specific knockdown experiments showed that Uzip function in glia, in addition to neurons, is also required for proper MB development. In order to test whether only glial Uzip expression is enough to rescue MB phenotypes, glia-specific rescue experiments with the pan-glial driver *repo-Gal4* in mutant background were performed.

Initially, Uzip was overexpressed only in glial cells in the homozygous null mutant background. These flies were still exhibiting very severe MB phenotypes as in null mutants (Figure 4.17 A-A’). So, glial expression of Uzip is not sufficient for the guidance of MB neurons. Next, the rescue was performed in the $uzip^{23}/uzip^{D43}$ transheterozygous background. As shown in section 4.3 these flies have low Uzip levels and exhibit very severe MB phenotypes, although the phenotypes are less severe than those of null mutants. Interestingly, overexpression of Uzip in glia of these transheterozygous flies results in efficient rescue of the MB phenotypes (Figure 4.17 B-B’).

Classification of the MB phenotypes observed in glial rescue experiements and quantification of the penetrance of each phenotype demonstrates the trend of reversing the wild type state (Figure 4.18).

4.6.4. Identification of Genetic Interaction Network of Uzip During Mushroom Body Development

A lot of molecules to regulate axon growth and guidance both autonomously and non-autonomously have been described. These axon guidance molecules include many CAMs, transcription factors, signalling molecules etc. and most of them are working together to regulate the complex behaviour of axons to ultimately generate precise wiring of the CNS (Chedotal and Richards, 2010; Raper and Mason, 2010). A subset of these molecules, are also known to regulate MB axon guidance as described in Section XXX. Identification of the position of a novel axon guidance molecule in the already existing gene network would give important insight about understanding the mechanism of how this novel molecule regulates the guidance process. In order to identify the interaction partners of Uzip, the

molecules that cause similar phenotypes in LOF studies and have a similar expression pattern were identified from the literature and the genetic interaction between these molecules was analysed.

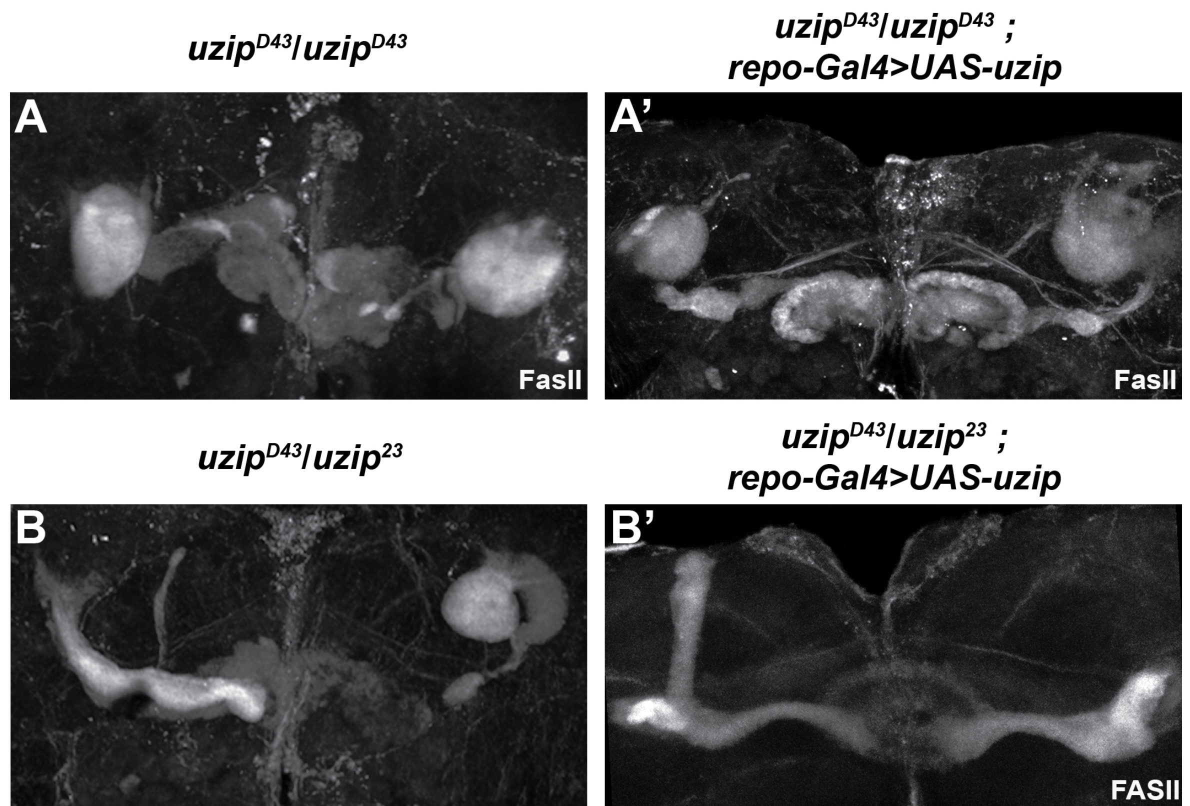

Figure 4.17. Rescue of glial Uzip in the mutant flies. (A) Null mutants exhibit axon stalling phenotypes and (B) overexpressing Uzip only in glia does not rescue these phenotypes. (C) Mutants transheterozygote for null and hyphomorph alleles still exhibit very severe phenotypes as thin lobes and axon stalling but in this background (D) overexpression of Uzip in glia decreases the severity of the phenotypes significantly.

The first candidate that was tested as a possible interaction partner was *neuroglian*. Nrg is a cell adhesion molecule known to regulate MB axon guidance (Goossens *et al*., 2011; Siegenthaler *et al*., 2015). It has two isoforms, one specific to glia and another that is specific to neurons. Its role in the development of the olfactory system, TIFR glia, and the MB are very similar to the one of Uzip; thus, it appeared as a very promising candidate for interaction. Flies hemizygote for *nrg*^{849} null allele exhibit axon-stalling phenotypes

(Goossens *et al.*, 2011), while heterozygote females have normal MBs. Interestingly, transheterozygote females for *nrg*^{849} and *uzip*^{D43} null alleles exhibit a lobe-missing phenotype (Figure 4.19 A-A'). These experiments show that Uzip and Nrg are genetically interacting during MB development.

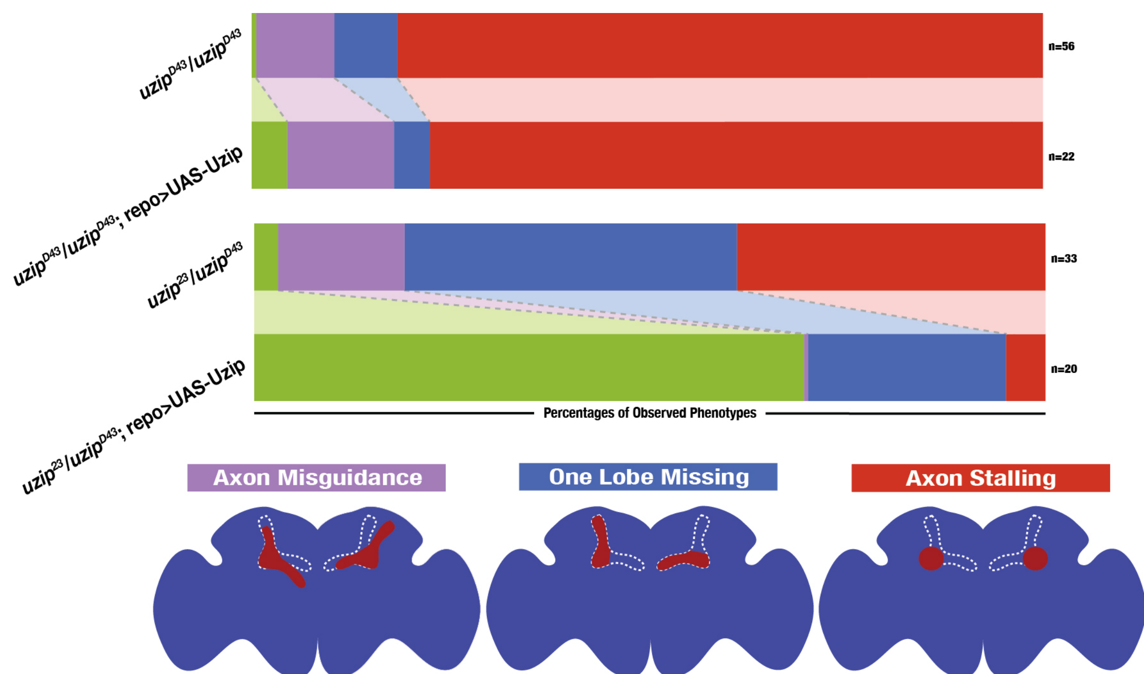

Figure 4.18. Quantification of MB phenotypes in glial rescue experiments. Introducing glial Uzip in null background does not result in any rescue in the phenotypes whereas in transheterozygote background most of the MBs resembles the WT. Green bars indicate wild type, purple bars axon misguidance, blue bars MBs with one lobe missing, and red bars MBs with axon crawling phenotypes. The length of each bar is proportional to the percentage of MBs observed with the corresponding phenotype among all investigated MBs of a certain genotype. Two hemispheres of each brain are scored independent of each other and n indicates the number of hemispheres examined. The schemes at the bottom of the graph depict the phenotypes represented by each category.

Another CAM important in axon guidance and neuronal circuit formation is *CadN*. During MB development it is expressed by developing Kenyon cells and mediates neurons

to generate bundles. In the absence of CadN, lobe missing and β lobe fusion phenotypes have been observed. Loss of CadN during development results in β lobe fused MBs and abnormally branched axons (Kurusu *et al*., 2012). Additionally, *uzip* and *CadN* have been shown to genetically interact during embryonic CNS development (Ding *et al*., 2011). Flies heterozygous for null allele *CadN*^{M12} have normal MBs, whereas transheterozygotes for null alleles of both *uzip* and *CadN* have MBs with missing and misshaped lobes (Figure 4.19 B-B').

Semaphorins are a very important and well-studied family of axon guidance molecules. *Sema-1a* genetically interacts with *nrg* during MB development (Goossens *et al*., 2011) and it is also required for proper TIFR formation (Zwarts and Callaerts, unpublished). In line with this information, the genetic interaction between *sema-1a* and *uzip* has been examined, and transheterozygotes for null alleles *sema-1a*^{K} and *uzip*^{D43} were shown to exhibit missing lobe phenotype, whereas the single heterozygotes have wild type MBs (Figure 4.20 A-A').

There are two genes that are known to regulate TIFR formation and maintenance, *nrg* and *drl*. *Drl* encodes for a receptor tyrosine kinase, and from the identification of the gene, a lot of different neural populations has shown to require *drl* for proper axon projection (reviewed in Clark *et al*., 2014). In the case of MB axon guidance, *drl* expressed from other cell lineages regulate the process indirectly (Reynaud *et al*., 2015). *Drl* null mutants display MBs collapsed on the midline (Grillenzoni *et al*., 2007) but heterozygous null mutants have normal MBs. Transheterozygotes for *drl*^{exc21} and *uzip*^{D43} null alleles exhibit β lobes fused phenotypes (Figure 4.20 B-B').

FasII is one of the very well charactarized CAMs for neural development. It is particularly important for MB development during metamorphosis. Requirement of *fasII* expression by α'/β' neurons for proper axon projections was shown and at later stages, α/β neurons are also expressing *fasII* to be able to follow the pioneer axon tracts (Fushima and Tsujimura, 2007; Kurusu *et al*., 2002). Adult α/β and γ neurons keep expressing FasII and immunohistochemistry with α-FasII antibody is the most common method for visualizing adult MBs. Thus, *fasII* is a possible candidate for interaction and identification of whether

there is an interaction is particularly important since α-FasII stainings had been used extensively in this study. Flies heterozygous for the null allele *fasII*^{EB122} exhibit normal MB morphology and double heterozygotes for this allele and the *uzip*^{D43} null allele also do not display any morphological phenotype in the MB (Figure 4.21 A-A').

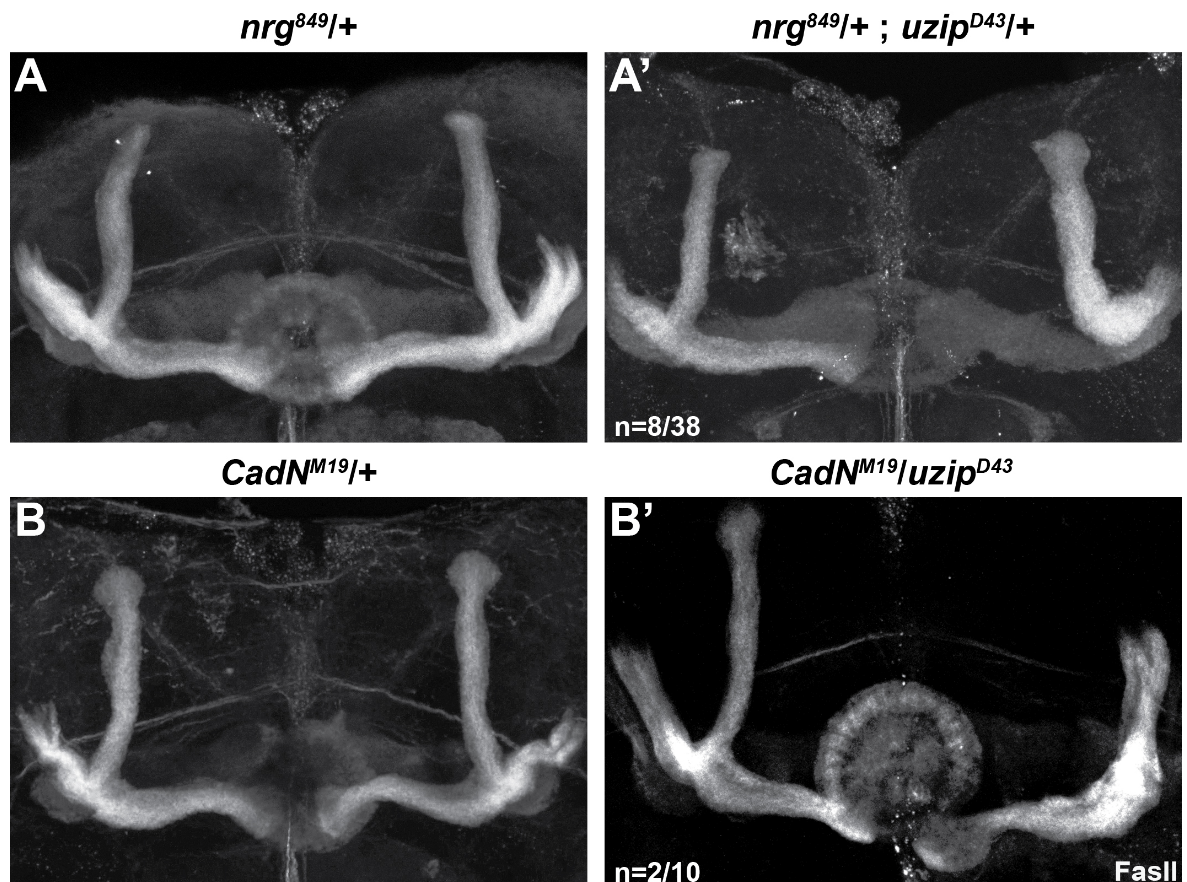

Figure 4.19. Analyses of genetic interaction of *nrg* and *CadN* with *uzip*.
(A) Heterozygozity for *nrg*^{849} or (B) *CadN*^{M12} null alleles does not result in any MB phenotype whereas double heterozygosity for (A') *nrg*^{849} and *uzip*^{D43} results in lobe missing and (B') *CadN*^{M12} and *uzip*^{D43} results in β lobe fusion phenotypes. n indicates the number of hemispheres examined.

Wnt is an evolutionarily very well conserved gene family encoding secreted glycoproteins. These genes are important in different aspects of development and particularly neural development (reviewed in Patapoutian and Reichardt, 2000). *Wnt5* is one of the very well characterized members of this family with respect to its role in CNS

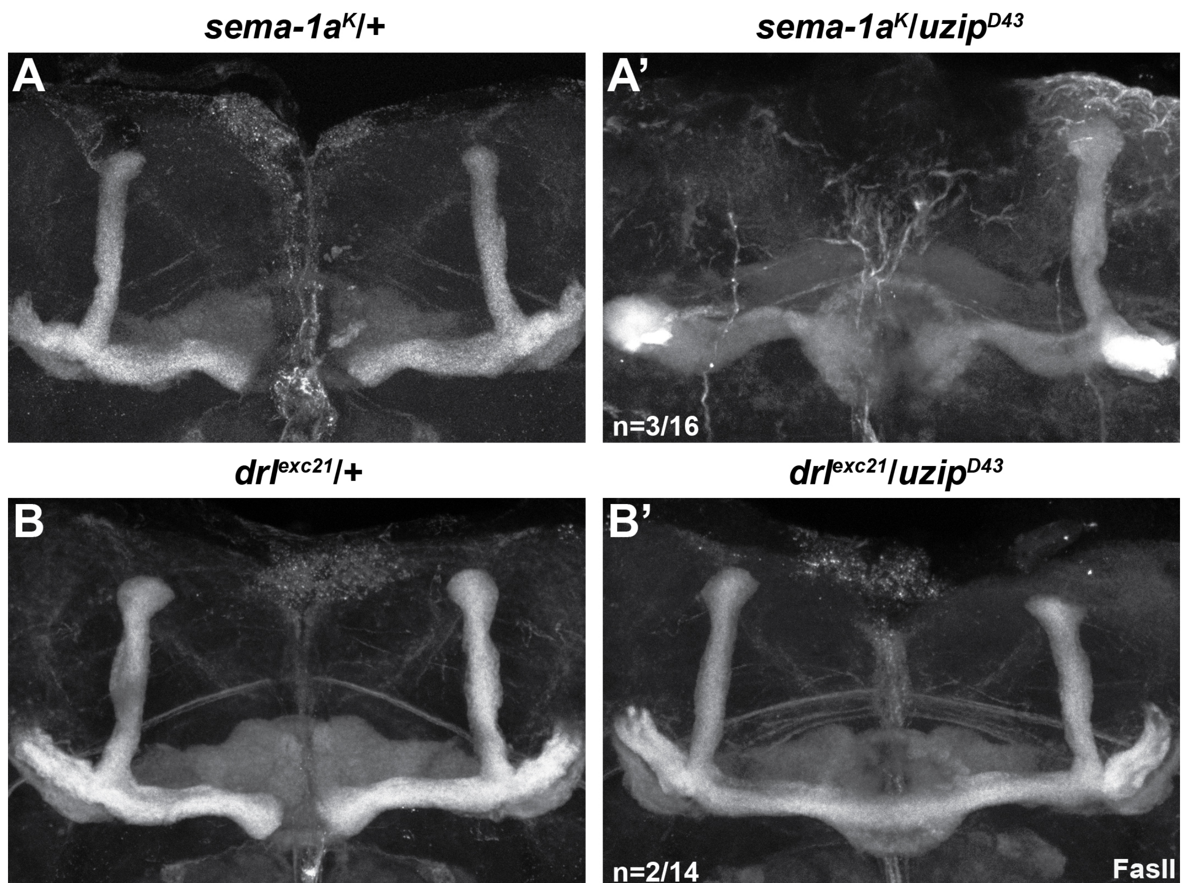

Figure 4.20. Analyses of genetic interaction of *sema-1a* and *drl* with *uzip.*
Single heterozygote flies for null alleles (A) *sema-1a*^{K} and (B) *drl*^{exc21} have vitually wild type Mbs, whereas transheterozygotes for (A') *sema-1a*^{K} and *uzip*^{D43} exhibit one lobe missing phenotype; (B') *drl*^{exc21} and *uzip*^{D43} exhibit β lobe fused phenotype. n indicates the number of hemispheres examined.

development. During MB development Wnt5 is acting as a ligand for Drl and planar cell polarity proteins to regulate MB development (Reynaud *et al*., 2015; Shimizu *et al*., 2011). *Wnt5* is also in the same genetic interaction network with *uzip* during embryonic CNS development (Ding *et al*., 2011). However, genetic interaction analysis between *uzip* and *wnt5* showed that both single heterozygous flies for *wnt5*^{400} null allele and double heterozygotes for this allele and *uzip*^{D43} null allele have wild type MBs (Figure 4.21 B-B'). Therefore, there is no evidence for the interaction between those two genes during MB development.

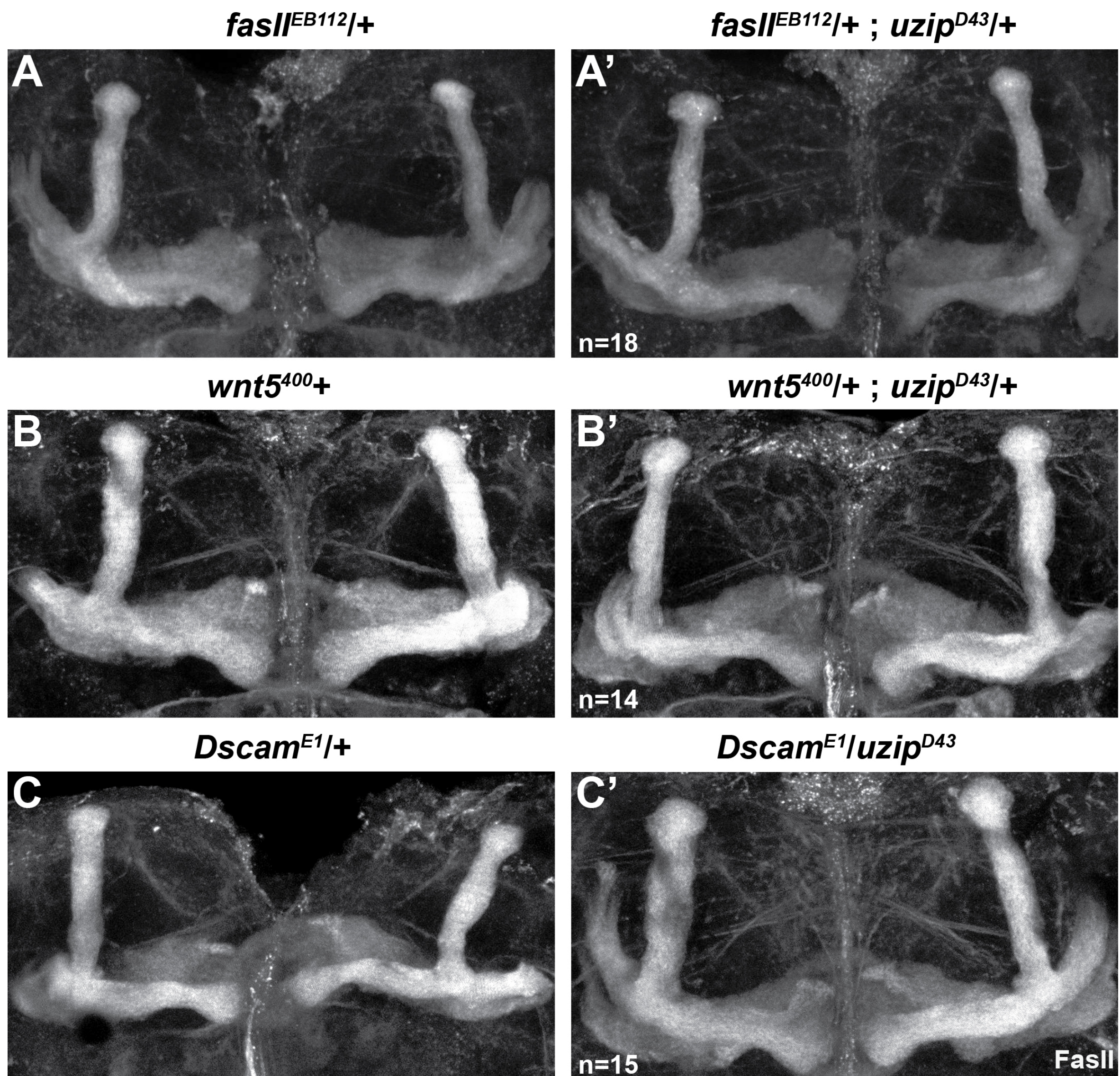

Figure 4.21. Anayses of genetic interaction of *fasii*, *wnt5* and *Dscam* with *uzip*. Flies heterozygous for null alleles of (A) *fasII*, (B) *wnt5,* or (C) *Dscam* exhibit no abnormality regarding the MB morphology and flies transheterozygous for null alleles of (A') *fasII* and *uzip,* (B') *wnt5* and *uzip,* or (C') *Dscam* and *uzip* also do not display any MB defect.

The last CAM tested for genetic interaction was *Dscam*. *Dscam* is an extraordinary gene, which encodes around 38,000 different mRNAs through alternative splicing (Celotto and Graveley, 2001). Loss of *Dscam* expression results in the same set of MB phenotypes observed in *uzip* mutants (J. Wang *et al*., 2002) and decreasing the Dscam diversity mimics the LOF phenotypes (Zhan *et al*., 2004). So far, there is no evidence showing *Dscam* is involved in a genetic interaction network with any other genes during MB development.

And here the MBs of individuals heterozygous for null allele *Dscam*^{E1} alone and together with *uzip*^{D43} null alleles display no phenotypes (Figure 4.21 C-C'). Thus, we conclude that *uzip* and *Dscam* are not in the same genetic interaction network.

Netrins comprise another important class of axon guidance molecules that are conserved very well throughout evolution. Netrins and their receptors are particularly important for midline crossing in the embryonic nervous system (Cate *et al*., 2016), control the target layer specifity of neurons in the visual system (Timofeev *et al*., 2012) and guide olfactory sensory neurons to the olfactory bulb (Lakhina *et al*., 2012). There are two *netrin* genes in *Drosophila* and the flies heterozygous for the null allele of both of the genes, *netrinAB*$^{\Delta}$ have morphologically normal mushroom bodies and double heterozygotes for this allele together with *uzip*^{D43} allele exhibit no abnormality in their MB morphology (Figure 4.22 A-A').

Trio is a Dbl family protein that activates Rho family GTPases. *Trio* has an important role in the developing embryonic nervous system as an axon guidance molecule and this role involves Drl, Unc5, and Robo (Long *et al*., 2016). Since Rho GTPases are shown to be key regulators in MB development and *trio* is also expressed very strongly by MB neurons and required for proper axon guidance of Kenyon cells (T Awasaki *et al*., 2000), it is a good candidate to test as a possible genetic interaction partner of *uzip*. Heterozygous flies for *trio* null allele *trio*^{1} exhibit no abnormality in terms of MB morphology and introducing the *uzip* null allele *uzip*^{D43} does not induce any axon guidance phenotype either (Figure 4.22 B-B').

The last gene that was tested for a possible genetic interaction with *uzip* is *Abl*. This gene encodes for a receptor tyrosine kinase and is a downstream effector of APP. In the absence of Abl in MB neurons the axons cannot project normally (Soldano *et al*., 2013). Heterozygozity for null allele *Abl*^{4} does not result in any axon projection errors in MBs and a combination of one copy of this allele and the *uzip*^{D43} null allele does not either (Figure 4.22 C-C').

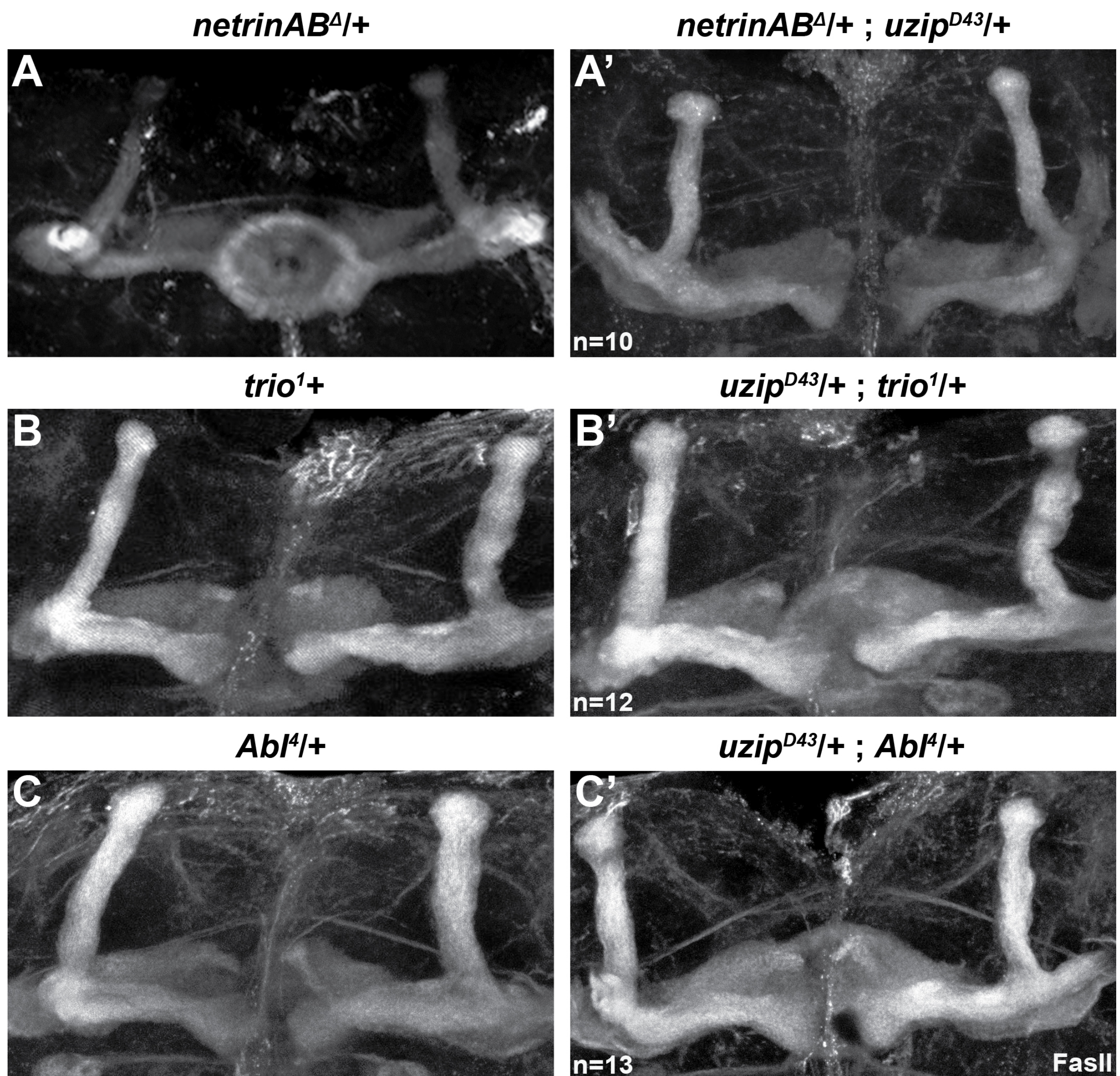

Figure 4.22. Analyses of genetic interaction of *netrinA*, *netrinB, trio* and *Abl* with *uzip*. Flies heterozygous for null alleles of (A) *netrinA* and *netrinB*, (B) *trio* or (C) *Abl* exhibit no abnormality regarding MB morphology and flies transheterozygous for null alleles of (A') *netrinA*, *netrinB* and *uzip,* (B') *trio* and *uzip,* or (C') *Abl* and *uzip* also do not have any MB defects.

5. DISCUSSION

Following the ground breaking neuroanatomical works of Ramón y Cajal who identified the morphology of nervous system cells the nervous system has become the limelight for a lot of scientists. Initial questions about the nervous system were related with how complex behaviours, emotions, capabilities of learning as well as physical control of the body are encoded within the cells and networks generated by them. After more than a century of research, thousands of books and publications the main revelation is that these initial naïve questions are actually the hardest ones to answer and we are still far from answering them. However, the initial general questions have diverged and became more specified on certain aspects of brain function. A lot of scientists are trying to contribute to the big picture of brain function by answering smaller but very important questions.

One of the most interesting questions among the many is how individual axons find their path during brain development. A lot of studies were conducted and revealed different aspects of this process, yet the overall picture is still patchy. Our study focuses on the regulation of axon guidance of the *Drosophila* MB neuropil. The MBs are a prominent part of the protocerebral brain segments present in most of the species of arthropod taxa (Strausfeld *et al*., 2009). In *Drosophila*, each MB consists of three different types of neurons and the final organization of the axons of those neurons generates five distinct lobes. All neurons in the MB are generated from the same neuroblasts in a sequential manner and their axons have different projection patterns (Lee *et al*., 1999). MBs are developmentally and anatomically well defined and as many genetic tools and techniques have been generated specifically to study this structure, they have become excellent models to investigate the dynamics of axon guidance in cellular and molecular level.

In the framework of this study, we aimed to investigate the role of the CAM Uzip in mediating distinct neuron-neuron and neuron-glia interactions in the development of the *Drosophila* MB. Following detailed expression pattern analyses the role of Uzip in proper

MB development was investigated through LOF and GOF experiments. Additionally, possible interaction partners of Uzip were identified through genetic interaction analysis.

5.1. Uzip is Required for Neuron-Glia Interactions During Development

Previous studies of Uzip function revealed that this protein is expressed in both glia and neurons during embryonic nervous system development and it coordinates axon guidance of the central nervous tract together with two other axon guidance molecules, CadN and Wnt5 (Ding *et al*., 2011). Studies in the visual and olfactory sensory systems show that Uzip is expressed both in neurons and glia in both of these systems. Its expression is required for axon guidance of photoreceptor and olfactory sensory neurons. Both expression patterns and detailed LOF experiments suggest that Uzip mediates proper axon targeting of those sensory neurons by mediating neuron-glia interactions during development (Kaan Mika, 2014; Ece Terzioğlu Kara, 2015).

5.1.1. Uzip is Expressed by Glia Associated with Mushroom Bodies

Three main tools were used to localize Uzip expression. The enhancer trap line *AC783-Gal4* inserted to the first intron of *uzip*, revealed an expression pattern that resembles glia associated with MBs when visualized with membrane-bound GFP.

Several lines of evidence supported the idea that Gal4 expression from this line partially or fully resembles the Uzip expression pattern. First of all, *uzip* is located in a gene rich region of the genome; there is another gene located ~100 bp upstream of the 5' UTR and ~600 bp downstream of the 3' UTR. It is very unlikely to fit regulatory regions that mediate such a complicated expression pattern in these regions. The second line of evidence is that flies homozygous for the AC783 allele exhibit MB phenotypes that phenocopy the full mutant phenotype, indicating that this insertion probably disrupts some regulatory elements. Lastly, the *uzip* BAC construct rescues the phenotypes completely, which means the gene regulatory regions are not located further away. In the light of all

those evidences, the gene regulatory regions of *uzip* are most likely located in the very big second intron, which is ~15kb.

The second tool used to localize Uzip was the Uzip::mCherry BAC transgenic fly line. Uzip::mCherry fusion protein was visualized around MB lobes but condensed around the heel region. Both ensheathing and astrocyte-like glia populations are located around the heel region. So, this expression pattern is mainly related to those cell types. OK107-driven membrane-bound GFP that labels all Kenyon Cell membranes did not seem to be co-localized with Uzip::mCherrry indicating that this fusion protein is not localized on adult MB neurons.

There are two glial subtypes that are tightly associated with adult MBs, ensheathing glia and astrocyte-like glia (Figure 1.3). These glial populations are known to have important roles in the development of MBs and are crucial during axon pruning (reviewed in Yu and Schuldiner, 2014). During metamorphosis, γ neurons go through extensive rearrangements, which sequentially include axon pruning, retraction, and projection events. Both of the glial subtypes are localized in the same regions, and at the adult stage both ensheathe the MB neuropil. The cells that are labelled not only by the enhancer-trap line *AC783* but also Uzip::mCherry appear to label the same glial populations. However, it is not possible to distinguish the glial populations since the morphology and spatial arrangements of glial populations vary from individual to individual. Thus, it is impossible at this point to determine the identity of the glia according to their localization or morphology (Doherty *et al*., 2009). Accordingly, it is not possible to decide whether either or both of these glial populations express Uzip.

The immunostaining with α-Uzip antibody generated in our lab did not reveal the localization of Uzip. The antibody was previously shown to recognize Uzip protein on Western blot analysis and in eye imaginal discs when Uzip was over-expressed (Ece Terzioğlu-Kara, 2015). However, in wild-type controls it did not work as efficiently. In adult brain staining, the localization was mainly observed on the brain surface indicating that the antibody is unable to penetrate the tissue. Using different protocols with different fixation methods for immunohistochemistry may help to improve the staining.

5.2. Uzip is Required for Path Decision of Kenyon Cell Axons

Kenyon cells do not migrate after they are born, but immediately start to develop and they project their axons and dendrites. A developing MB axon has several decision-making points. The first one is to get out of the region of cell bodies and dendrites, the calyx, and enter the initial axon bundle, the peduncle. After this initial decision, each axon projects through the peduncle as they are encircled with previously projected axons. At the end of the peduncle, every axon bifurcates and generates two sister axon branches. After the proper bifurcation the next challenge is to separate the two sister axon branches from each other, one projecting dorsally and the other medially. If this final projection is also finished properly each MB neuron subtype generates two distinct lobes. The neurons born during embryonic stages – γ neurons – go through axon pruning and regrowth during pupal stages to generate a single lobe, whereas the later-born two neuron subtypes do not undergo axonal rearrangements (Lee *et al*., 1999).

The analyses of γ neuron morphology in *uzip* mutants revealed that axonal projections of those neurons are not affected. The only observed phenotype is that γ lobes are slightly thinner than the wild type. This is more likely to be the result of alteration of the morphology of the other MB neurons. The projection pattern of γ neurons were unaltered even in MBs with axon crawling phenotypes in α/β and α'/β' neurons. However, this is difficult to observe with a α-FasII staining, because γ neurons are only stained lightly, but it is very obvious in OK107-driven knockdowns (Figure 4.10). These observations indicate that Uzip is not involved in the projection of γ neurons. Analyses of MBs of *uzip* mutants performed at 3rd instar larval stage support this hypothesis. At this stage the MBs mainly consist of γ neurons and in mutants no phenotypes were observed. Together with the previous studies on MB axon guidance, it can be suggested that the earliest born γ neurons have a different mechanism for axon projection than the later born neurons.

The most severe phenotype observed in the mutants was axon crawling. This phenotype has resemblance to previously observed axon-stalling phenotypes of *rac* family actin regulator mutants (Ng *et al*., 2002) and axon-crawling phenotype of *nrg* mutants

(Siegenthaler *et al*., 2015). In the first case the axons stall around the entrance of the peduncle and cannot project further, whereas in the latter case the axons cannot enter the peduncle but they grow where they are and generate ball-like structures. The localization of the ball-like structures observed in this study and also their size suggests that, *uzip*-deficient axons cannot enter the peduncle, yet they continue to grow and crawl around the calyx region. Thus, Uzip is required for α/β and α'/β' axons to enter the peduncle not for their growth.

In none of the *uzip* mutants any axon stalling was observed throughout the peduncle stalk, which means that if the axons can pass the initial decision point and enter the peduncle, they follow it all the way until the end. At the end of the peduncle, the axons need to bifurcate and two branches need to project to different regions. In general, lobe-missing phenotypes can arise through two different ways: either the axons do not branch, or the branches do not separate from each other and follow the same path. When the lobe missing phenotype of milder mutants was investigated, it became obvious that when one lobe is missing the remaining lobe was getting thicker than the controls indicating that *uzip* deficiency does not interfere with the bifurcation process, rather the sister axon branches cannot separate from each other. And when the α or β lobe-missing phenotypes were quantified individually, no bias on loss of either of the lobes was observed to be greater than the other so the role of Uzip in lobe formation is not lobe-specific.

MB axons in *nrg* mutant animals bypass the peduncle and the lobes to directly project to the α lobe tip (Siegenthaler *et al*., 2015). This observation shows that there are some long-range cues around this region that attract the axons. The short lobes observed in *uzip* mutants result from axons that stop prematurely. This indicates that in *uzip* mutants these cues are missing or mislocated. Considering the localization of Uzip around the MBs and the fact that Uzip has a secreted isoform this long-range cue may be Uzip itself. The crooked projection phenotypes probably result from mislocation or lack of the Uzip signal so that the axons project to some other directions.

The third phenotype observed in *uzip* mutants is thin lobes. MB axons project *en masse*, and there are very strong interactions between the axons and previous studies

suggest that the physical forces between axons are the main force driving the MB axon projection (Kurusu *et al*., 2012; Siegenthaler *et al*., 2015). However, whenever a thin lobe phenotype is observed in *uzip* mutants, the other lobe generated by the same neurons is getting thicker. These observations suggest that some of the axons cannot separate from their sister axons so thin lobe phenotype is a semi-penetrant version of lobe missing phenotype.

A possible scenario for thin lobes is that the thin lobes only comprise α/β pioneer neurons, and that the follower axons cannot follow the pioneers in the absence of Uzip. In order to test this hypothesis knockdown experiments with *c708-Gal4* (specifically expressed in α/βp neurons) was performed but it did not result in any axon guidance phenotypes. Besides, there is roughly the same number of α/βp neurons in each individual (Lee *et al*., 1999), whereas the thicknesses of the observed lobes vary significantly. Thus, the role of Uzip during MB axon guidance mediating is neither the projection of the pioneer neurons nor the interactions between pioneer and follower neurons.

In the light of data coming from the localization and mutant analyses we postulate the scenario that Uzip is localized along the MB axon tract and the axons follow this Uzip cues to project normally. If Uzip is absent initially around the entrance of the peduncle axons cannot enter the peduncle, which results in the axon crawling phenotype. Then when Uzip levels are low, the axons compete for the Uzip localized on the tract and this leads to thin lobe or in more severe cases lobe-missing phenotypes. This model can explain all phenotypes observed in mutants and is also consistent with the dose-dependence of the phenotypes.

5.3. Mushroom Body Neurons Need to Express Uzip for Proper Axon Guidance

Our previous hypothesis states that Uzip is localized along the path of MB axons and the axons follow this Uzip cue to project properly. There are two critical steps to prove this hypothesis; we had to know how Uzip is localized along this tract and how the neurons are able to follow this cue. Since Uzip is forming homophilic interactions, it is possible that the axons are also expressing Uzip and this allows them to follow the path.

Initial experiments with *elav-Gal4* (*c155-Gal4*) and *UAS-UzipRi* (TRiP), resulted in lobe missing phenotypes that is consistent with *uzip* mutant phenotypes. However, this line is known to mediate Gal4 expression in all CNS neurons and some glial cells (Grillenzoni *et al*., 2007; Berger *et al*., 2007). Some neuronal types and also this Gal4 positive glia may be responsible for localization of Uzip to the MB axon tract, so in order to restrict Uzip knockdown to only MB neurons the *OK107-Gal4* driver was used.

Knockdown experiments performed with the OK107-Gal4 driver resulted in lobe-missing and other axon misguidance phenotypes. These data confirmed that Uzip expression in MB neurons is required for normal axon guidance. Enhancing the knockdown of Uzip via overexpression of Dcr2 increased the severity and the penetrance of phenotypes, as the same trend was observed in mutants.

These results are consistent with our hypothesis of Uzip function during MB axon guidance and we extended our hypothesis. Uzip is localized along the MB axon trajectory path and Uzip-positive MB axons are able to follow this path by means of homophilic Uzip interactions. When the Uzip levels on the MB axons are decreased they are unable to follow this path and generate thin, misguided or missing lobe phenotypes and axon crawling in the severe cases. γ neuron axon guidance is not dependent on Uzip since the formations of γ lobes are unaffected in the absence of Uzip.

5.4. Uzip Mediates Interactions Between Mushroom Body Neuron Subtypes

The second important part of the hypothesis of Uzip function is to identify which cells are responsible for the localization of Uzip on the path of MB axons. The best candidate for that were the previously projected MB axons. Given the fact that α'/β' neurons are born before γ neuron axon pruning and γ neuron axon projection is independent of Uzip, we can speculate that the two lobes generated by γ neurons before pruning localize Uzip and newly born α'/β' follow them by expressing Uzip. After those neurons project and form α' and β' lobes, the latest born α/β neurons follow α'/β' neurons again via Uzip expression. Initial observation of all MB morphology in knockdown lines is

consistent with this hypothesis. It was observed that in some brains α/β neurons could not project properly even though α'/β' axons projected normally (Figure 4.10).

MB neuron subtype-specific knockdown experiments revealed that, when Uzip expression was decreased only in γ neurons it did not result in any MB defects. This data disproved the hypothesis that γ neurons guide α'/β' neurons via Uzip expression. However α'/β' neuron specific knockdown experiments resulted in loss of α and β lobe missing phenotypes. Likely, decreasing Uzip expression only in α/β neurons also resulted in α and β lobe missing phenotypes.

The latest-born α/β neurons project their axons through early-born α'/β' axon tracts (Siegenthaler *et al*., 2015). Failure of α' or β' lobe development results in failure of α or β lobe development since their guidance cue is not present. There are several studies that identified molecules controlling α and β lobe formation indirectly by guiding α' and β' lobes (Goossens *et al*., 2011; Ng *et al*., 2002). Uzip LOF in α'/β' neurons resulted in axon guidance defects in α/β neurons. This may be the result of either the lack of Uzip in α'/β' neurons causing the α/β neurons to project incorrectly as well since they have lost their guidance cue, or even if α'/β' neurons project normally α/β neurons may not be able to follow them. α/β neuron-specific knockdown experiments are favouring the second scenario since α'/β' neurons are expected to project normally in these lines, but still lobe missing phenotypes were observed. The previous observation mentioned above in which α/β neurons may not project properly even though α'/β' axons extend normally is also consistent with this scenario.

Taken together the previous observations in which α'/β' neurons guide the α/β neurons and our observations, we can say that Uzip is required for α/β neurons so that they can follow α'/β' axon tracts and project normally.

5.5. Glial Uzip is Required for Mushroom Body Axon Guidance

Our initial hypothesis states that Uzip is localized around the MB region and α'/β' and α/β neurons follow this path by expressing Uzip. Functional studies performed on MB

neurons confirmed that Uzip mediates axon-axon interactions and those interactions mediate the projection of the latest-born α/β neurons. However, which cells express Uzip to serve as guidance cue for α'/β' neurons is still unknown.

Uzip is mainly expressed by glia both in the embryonic and adult CNS. In this study we show that glia associated with MBs express Uzip. Considering this, we speculated that glia might guide MB axons by expressing Uzip. Glial Uzip knockdown experiments with pan-glial *repo-Gal4* driver revealed that in the absence of glial Uzip MB axons cannot project normally. Initial experiments with the *repo-Gal4* enhancer trap line did not result in any phenotypes. This is due to the insufficient Gal4 expression from this transgene (Christian Klambt, personal communication). However, when two *repo-Gal4* transgenes are combined it results in lobe missing and α lobe overgrowth phenotypes.

One of the phenotypes observed in glial knockdown experiments is α lobe overgrowth and this phenotype arises when axons are unable to terminate their projection at their regular target region. This phenotype observed in glial knockdown experiments suggests that glial Uzip expression is important for axon growth termination. Taken together with the Uzip expression profile, it can be speculated that Uzip is expressed around MB lobe tip regions by glial cells, and MB axons interact with these glia through homophilic Uzip interactions. The adhesive force generated between the two cell types most likely prevents axons to project further. However, it can be due to an experimental artefact as it has been observed in only one of the 20 samples visualized.

Lobe missing phenotypes observed in glial knockdown experiments support our hypothesis that glia are guiding the MB axons. When glia do not express Uzip the axons cannot follow the glia anymore and lobe missing phenotypes are observed. Pan-glial knockdown experiments do not give any information about the glial population responsible for the guidance of MB axons. Considering the expression pattern of Uzip, the best candidate is MB associated glia.

5.5.1. Uzip Loss-of-Function in MB Associated Glia does not Effect Mushroom Body Development

MB-associated glia were defined as ensheathing and astrocyte-like glia (Ou *et al*., 2016). Both of the glial cell populations function as phagocytes during CNS development. Ensheathing glia function as phagocytes mainly during antennal lobe development whereas astrocyte-like glial cells induce γ neuron pruning as well as engulf the axonal debris afterwards (Doherty *et al*., 2009; Hakim *et al*., 2014). Both of the glial subtypes are located around the MB neuropil throughout development and at the adult stage. However, even though these glial cell populations are tightly associated with MB axons it is not known whether they regulate axon guidance or if MB neurons are important for glial cell development (Gai *et al*., 2016; Ou *et al*., 2016).

Uzip knockdown experiments in either astrocyte-like glia or in ensheathing glia did not result in any axon guidance defect in MBs. However, this data is not enough to conclude that MB associated glia are not required for axon guidance since the results can be false negative. There could be three reasons for getting false negative results. There could be a Gal4 insufficiency as was experienced with the *repo-Gal4* transgene. However, here there is no other transgene to enhance the Gal4 levels. The other possibility according to our hypothesis is for these glial populations to express Uzip during 3rd instar larval stage to guide α'/β' neurons. However, the Gal4 driver transgenes were mainly identified for their Gal4 expression at adult stage. Maybe those cells do not express the Gal4 in enough levels or not at all during the larval stages. If so, Uzip knockdown did not take place when the axons projected so the process is not defective. The last possible reason is if both of those glial populations are expressing Uzip to guide MB axons, the other cell population might compensate the loss of Uzip in one population. Overall, the Gal4 expression profile of the Gal4 driver lines should be investigated at different developmental points. Knockdown experiments with both of the drivers at the same time or different drivers such as *nrv2-Gal4* should be performed.

5.5.2. Uzip Expression TIFR Glia is Required for TIFR-MB Interactions

In pan-glial knockdown experiments enhancing the RNAi activity by over-expressing *Dcr2*, as it was done in neurons, resulted in a β lobe fusion phenotype. This phenotype has never been observed either in mutants or in previously discussed knockdown experiments. This result does neither support nor disprove our hypothesis of Uzip function; it indicates a different mechanism that is probably independent of the one we have discussed so far.

Ablation of TIFR structure results in loss of α lobes and merge of all MB axons on the midline. It has important roles in regulating the position of MB lobes relative to the midline and also in commissure formation of OSN (See Section 1.3.2). Additionally, the TIFR structure is malformed in *uzip* mutants and this could be the possible cause of the failure in OSN commissure formation in these mutants (Kaan Mika, 2014). However, since the structure is not totally deformed, rather only in the ventral part it is not known whether Uzip deficiency of TIFR prevents it from exerting its repellent role on MB axons.

Uzip knockdown in TIFR glia results in β lobe tip outgrowth and β lobe fusion. This phenotype resembles the TIFR ablation phenotypes although phenotypes appear to be milder; here, the α lobes are unaffected. Increasing the activity of *RNAi* via *Dcr2* over-expression results in an increase in the phenotype and leads to the collapse of α lobes onto the midline. These data suggest that Uzip is required for proper TIFR formation in a dose-dependent manner: as the Uzip levels are decreasing the structure of the ring is getting more and more disrupted and it does not repel MB axons anymore.

This data indicates that Uzip is required for TIFR formation in a dose-dependent manner and when TIFR glia is Uzip deficient they cannot exert their repellent effect on MB axons. This situation raises the question whether TIFR glia could be the glial subtype guiding MB axons, as well? This is very unlikely because the TIFR is formed ~15 hours after pupal formation when most of the axons generating α' and β' lobes have already projected to their final locations. As a result, this mechanism is most likely to be

independent from the mechanism we have discussed so far and it represents an indirect role of Uzip on MB development.

5.6. Uzip Expression in Both Neurons and Glia is Required for Mushroom Body Axon Guidance

The loss-of-function analyses gave a lot of information about Uzip function during MB axon guidance. The reverse approach, complementing these experiments, is a molecular rescue experiment in which the *uzip* expression is restored in *uzip* mutant backgrounds. The expectation is to reverse the phenotypes observed in mutants back to the wild type state. This observation would prove that the phenotypes observed in mutant flies result from perturbation of the expression of the gene of interest.

The first rescue experiment performed with the uzip BAC lines resulted in complete rescue of the MB axon guidance defects. The BAC lines were generated via integration of the whole genomic locus of *uzip* – including ~1.5kb upstream and ~2.4kb downstream of the gene – into the genome at a different chromosome than the one on which *uzip* resides (Kaan Mika, 2014). This construct is considered to include all gene regulatory regions of *uzip* so the Uzip expression from the BAC construct is expected to have the same pattern and timing of expression as the endogenous gene. The complete rescue proves two points; first the BAC construct has similar expression pattern with endogenous gene and second, the phenotypes observed in the mutants are due to Uzip deficiency.

Two lines were generated with BAC transgenesis. The first one has the endogenous gene locus and the second one has a mCherry coding sequence inserted just before the stop codon of *uzip*. This second construct is expressing Uzip::mCherry fusion protein and since expression of this protein also rescues the mutant phenotypes, the localization of this fusion protein should be the same with endogenous Uzip.

The BAC rescue experiments also support the idea that *AC783-Gal4* line mimics the *uzip* expression. The BAC construct has ~1.5kb upstream of the gene and ~2.4kb downstream of the gene. Since the two other genes are residing within these regions, the

gene regulatory regions are not expected to reside in those regions. The most possible location is again the second intron of *uzip,* which is ~15kb in size and where the transposon is located in the *AC783-Gal4* line.

5.6.1. Neuronal Uzip is Enough to Rescue Mushroom Body Phenotypes

After rescuing Uzip expression in all the cells by introducing the BAC transgene, cell type-specific rescue experiments were performed with cell type-specific Gal4 driver lines and a *UAS-Uzip* construct that was previously generated in our lab (Kaan Mika, 2014). The initial attempts to rescue Uzip in all neurons with *elav-Gal4* were inconclusive. No progeny with the desired genotype could be obtained and thus a different approach for rescuing the expression only in neurons was used. A ubiquitous driver was combined with *UAS-Uzip* and *repo-Gal80* constructs in homozygous *uzip*^{D43} background. The most common and reliable ubiquitous driver for such studies is *Act5c-Gal4*. However, the combination of this driver with *UAS-Uzip* caused lethality. As a result, again a ubiquitous but less strong driver *da-Gal4* line was used. This experiment resulted in a striking rescue of MB axon guidance although the MBs did not look exactly like the wild type ones.

It is difficult to obtain a morphologically perfect rescue in which the MB phenotypes are reversed to wild type, since the rescue experiment is performed with a binary expression system. Uzip is, most probably, not a pan-neuronal protein but in the rescue experiment it was expressed in all neurons, which may be the reason for the abnormal morphology of the MBs. The other possible reason is that with this binary expression system Uzip is expressed constitutively in all neurons whereas the endogenous expression is most likely to have a more dynamic pattern.

On the other hand, according to our hypothesis that states that expression of Uzip by both cells types allows glia to guide MB axons, we expected to observe an incomplete rescue since glial Uzip is missing in these rescue experiments. This is most probably due to the pan-neuronal over-expression of Uzip: some neuronal lineages other than α’/β’ and α/β neurons might compensate for the loss of glial Uzip. For example, when Uzip is over- or ectopically expressed in γ neurons they may serve as a guidance cue for later born MB

axons, as we initially thought. Or, there is a neuronal cell lineage that has been shown to guide later born MB axons via expressing *drl* receptor. This neuronal lineage could serve as a guidance cue when it over/ectopically expresses Uzip. In order to overcome this problem, rescue experiments with MB-specific *OK107-Gal4* driver line were performed, but no progeny with the desired genotype could be obtained. Because of time limitations these experiments could not be finished in the framework of this study. However, rescuing expression only in Kenyon cells could be tried with a different MB-specific driver such as *Mef2-Gal4*, which would be easier because of technical limitations of the *OK107-Gal4* driver.

5.6.2. Only Glial Uzip Expression is not Sufficient To Rescue Mushroom Body Axon Guidance Defects without Neuronal Uzip

The previously discussed data show that the glial Uzip expression is required for proper MB axon guidance. The first glial rescue experiments showed that in *uzip* null mutant background rescuing the glial expression of Uzip is not enough for rescuing MB axon guidance defects. On the other hand, when the rescue was performed in $uzip^{23}/uzip^{D43}$ transheterozygous background, a significant rescue of the phenotypes was observed.

These results strengthen the hypothesis that glial cells are guiding the MB axons and Uzip expression is important for this guidance. According to our hypothesis, a projecting α’/β’ or α/β axon should express Uzip so it can follow the tract that is generated by localized Uzip. Since *repo-Gal4* driven rescue does not provide Uzip expression in the MB neurons and there is no Uzip expression in those neurons in null mutant background, these neurons cannot project even if the tract is generated by Uzip localization by glia. However, in trans-allelic combinations of null and hypomorph alleles, these neurons have low levels of Uzip expression. And when the Uzip is over-expressed in glia, Uzip is localized on the tract of the axons again and they can project normally.

To sum up, our current hypothesis of Uzip function during MB axon guidance is as follows: Uzip is expressed by axons of α’/β’ neurons and they follow Uzip positive glia to project to their final locations. Later-born α/β neurons, also expressing Uzip, project to

previously formed α’/β’ axon tracts and/or the same Uzip-positive glia. Localizing Uzip to the regions of MB lobes by some cell populations may compensate for the loss of glial loss of Uzip, however expression by the α’/β’ and α/β axons is necessary for their proper projection.

5.7. Uzip Signaling During Mushroom Body Axon Guidance Includes Previously Identified Axon Guidance Molecules

Our hypothesis focuses on homophilic interactions between Uzip proteins localized both on the outgrowing axon and the substrate it is following. However, axon guidance mechanisms mediated by *uzip* may involve some other molecules. In order to investigate this possibility, we aimed to identify the interaction of *uzip* with genes encoding axon guidance molecules known to be involved in MB axon guidance.

The initial step in the identification of possible interaction partners of a protein is to look at the literature to find others that have similar functions, expression patterns, and/or cause similar phenotypes in LOF experiments. While there are several ways to test the interaction between two molecules, the most straightforward method that allows screening of a lot of molecules in a short time is genetic interaction screening. This method aims to identify whether two molecules are working in the same pathway in a given developmental process.

Axon guidance molecules generally do not exhibit haploinsufficiency, so heterozygosity for a null allele of an axon guidance molecule does not cause any developmental phenotypes. However, transheterozygosity for two genes that exert their function through the same signalling pathway has a much higher chance to cause a phenotype (Mani *et al*., 2008), a situation termed as co-induced haploinsufficiency. The genetic interaction analysis revealed that there are co-induced haploinsufficiencies between *uzip* and *nrg*, *CadN*, *sema-1a*, and *drl*.

5.7.1. *Uzip* is Involved in the Same Genetic Interaction Network with *nrg* and *sema-1a* to Control MB Axon Guidance

Nrg encodes for a CAM that has a very important role in axon guidance of different neuron populations in the CNS. The protein has two isoforms one of which is glia and another one that is neuron-specific. During MB axon guidance late born neurons follow the early born ones through membrane-localized Nrg on both types of neurons. Nrg expression is both required for axons to enter the peduncle and later for formation of the lobes. However, there is no study investigating the role of glial nrg in MB development. Apart from that *nrg* is one of the central genes controlling TIFR formation.

Both Uzip and Nrg are forming only homophilic interactions with their extracellular domain as far as known. Accordingly, Co-IP experiments performed between those two proteins showed that there is no interaction between the two proteins (Patrick Callaerts, unpublished). Together these data indicate that, *Nrg* and *uzip* may function through the same signalling pathway during MB development without any physical interactions between the proteins.

The semaphorin family is composed of evolutionary well-conserved axon guidance molecules. Different members of the family mediate axon guidance through forward and reverse signalling (Battissini and Tamagnone, 2016). During MB axon guidance one of the genes from this family, *sema-1a*, is involved in a genetic interaction network with *nrg* (Goossens *et al*., 2011). Besides, *sema-1a* is also important for TIFR formation (Liesbeth Zwarts, personal communication). Genetic interaction experiments between *sema-1a* and *uzip* show that during MB axon guidance, *uzip*, *nrg*, and *sema-1a* are involved in the same genetic interaction network both in neuron-neuron interactions and during TIFR formation.

5.7.2. *Uzip* Signaling During the Mushroom Body Axon Guidance does not Include *Wnt5* but Its Receptor *drl*

Wnt5 genetically interacts with *uzip* to control axon guidance of the embryonic nervous system (Ding *et al*., 2011). This protein is involved in a lot of different

developmental processes including axon guidance (Inestrosa and Varela-Nallar, 2015). *Drl* encodes for a receptor for Wnt5 and the Wnt5/Drl pathway is known to be very important for olfactory system and MB axon guidance (Grillenzoni *et al*., 2007; Reynaud *et al*., 2015; Yao *et al*., 2007). *Drl* has also a central role in TIFR formation (Simon *et al*., 1998).

Drl is not expressed by MB neurons but its expression by another neuronal lineage is coupled with Wnt5 and guides MB axons to form lobes. Genetic interaction between *uzip* and *drl* indicated that, Uzip also participates in this signalling network. Even though no interaction between *wnt5* and *uzip* was observed, they must be genetically interacting through *drl*.

Co-induced haploinsufficiency of *drl* and *uzip* results in β lobe fusion phenotypes. This indicates that the interaction is taking place either during TIFR development or it is important for the interaction between MB neurons and the Drl-positive neuronal lineage that guides MB axons.

5.7.3. *CadN* is Involved in *uzip* Signaling During Musroom Body Development

CadN is involved in MB axon guidance, and genetic interaction analysis suggests that it is in the same signalling cascade with Uzip, as it is in axon guidance of the embryonic CNS. The lobe missing phenotype observed in trans-heterozygous individuals suggests that the two genes are involved in neuron-neuron and/or neuron-glia interactions to ensure MB axon guidance.

Previous studies showed that β lobe fusion phenotype have been observed in some *CadN* mutants, and the researchers hypothesized that in these mutants, the interactions between MB axons are not strong enough so they exhibit outgrowth phenotypes (Kurusu *et al*., 2012). In this study, three different experiments resulted in β lobe fusion phenotypes; glial knockdown of *uzip* enhanced with Dcr2 expression, TIFR knockdown and genetic interaction analysis with *drl*. In none of these experiments β lobe outgrowth only from one of the MBs was observed, even though axon guidance of the two MB lobes are independent. This indicates that this phenotype is related with a single mechanism that

prevents outgrowth of both of the β lobes at the same time and this mechanism most likely has to be pre-defined TIFR-MB interactions. Consequently, CadN may also be involved in TIFR development and involved in the same signalling pathway with Uzip in this process, as well.

5.7.4. Other Genetic Interaction Candidates

Co-induced haploinsufficiency experiments did not indicate genetic interactions between *uzip* and *fasII*, *Dscam*, *netrins*, *trio* or *Abl*. The method to look at genetic interactions between two proteins is one of many possibilities. Even though observing a phenotype in trans-heterozygote individuals indicates a genetic interaction, the opposite situation does not mean there is no genetic interaction. Other methods, such as epistasis analysis can be used to identify those interactions.

Overall, genetic interaction analyses can only give an idea to select candidates for further, more precise, interaction analyses. The interactions between candidates should be performed by more informative experimental settings such as CoIP or *in vivo* analyses such as FRET.

6. CONCLUSION

At the initial phase of this study, it was observed that Uzip is localized around MB neuropil at the adult stage. The cells that express Uzip are most likely to be the neuropil glia subtypes that are associated with MBs, namely ensheating and astrocyte-like glia.

Analyses of LOF mutants revealed that Uzip is required only for axonal path decision of later born MB neurons. In the absence of Uzip the axons grow but cannot follow their regular path. The early born γ neurons are unaffected.

Cell type specific LOF experiments displayed that MB neurons should express Uzip to be able to project their axons properly. Uzip mediates this process via regulating previously identified axon-axon interactions between α'/β' and α/β neurons. Glia specific LOF studies also resulted in MB axon guidance defects and this is the first time that glia are shown to be regulating MB axon guidance.

Molecular rescue experiments confirmed that both neuronal and glial uzip is required for proper MB axon guidance. Neuronal overexpression of Uzip can mask the phenotypes resulting from glial LOF in a great extense, however the reverse scenario is not true. The glial Uzip is not enough for axons to project normally in the absence of neuronal Uzip.

Finally, Uzip signalling during the MB axon guidance includes previously identified axon guidance molecules. *Nrg*, *sema-1a* and *CadN* are working together to regulate axon-axon or axon-glia interactions during MB development. On the other hand, *drl* and *uzip* are together regulating TIFR formation or TIFR-MB interactions.

REFERENCES

Awasaki, T., S.-L. Lai, K. Ito, and T. Lee, 2008, "Organization and Postembryonic Development of Glial Cells in the Adult Central Brain of Drosophila.", *The Journal of neuroscience: the official journal of the Society for Neuroscience*, Vol. 28, No. 51, pp. 13742–53.

Awasaki, T., M. Saito, M. Sone, E. Suzuki, R. Sakai, K. Ito, and C. Hama, 2000, "The Drosophila Trio Plays an Essential Role in Patterning of Axons by Regulating Their Directional Extension.", *Neuron*, Vol. 26, No. 1, pp. 119–131.

Bate, C. M., 1976, "Pioneer Neurones in an Insect Embryo.", *Nature*, Vol. 260, No. 5546, pp. 54–6.

Bates, K. E., C. S. Sung, and S. Robinow, 2010, "The Unfulfilled Gene Is Required for the Development of Mushroom Body Neuropil in Drosophila.", *Neural development*, Vol. 5, No. 4, pp. 1-13.

Berger, C., S. Renner, K. Lüer, and G. M. Technau, 2007, "The Commonly Used Marker ELAV Is Transiently Expressed in Neuroblasts and Glial Cells in the Drosophila Embryonic CNS", *Developmental Dynamics*, Vol. 236, No. 12, pp. 3562–3568.

Brochtrup, A., and T. Hummel, 2011, "Olfactory Map Formation in the Drosophila Brain: Genetic Specificity and Neuronal Variability.", *Current opinion in neurobiology*, Vol. 21, No. 1, pp. 85–92.

Cate, M.-S., S. Gajendra, S. Alsbury, T. Raabe, G. Tear, and K. J. Mitchell, 2016, "Mushroom Body Defect Is Required in Parallel to Netrin for Midline Axon Guidance in Drosophila", *Development*, Vol. 143, No. 6, pp. 972–977.

Celotto, A. M., and B. R. Graveley, 2001, "Alternative Splicing of the Drosophila Dscam Pre-mRNA Is Both Temporally and Spatially Regulated", *Genetics*, Vol. 159, No. 2, pp. 599–608.

Chedotal, A., and L. J. Richards, 2010, "Wiring the Brain: The Biology of Neuronal Guidance.", *Cold Spring Harbor perspectives in biology*, Vol. 2, No. 6, pp. 1–18.

Chen, W., and H. Hing, 2008, "The L1-CAM, Neuroglian, Functions in Glial Cells for Drosophila Antennal Lobe Development.", *Developmental neurobiology*, Vol. 68, No. 8, pp. 1029–45.

Cho, W., U. Heberlein, and F. W. Wolf, 2004, "Habituation of an Odorant-Induced Startle Response in Drosophila.", *Genes, brain, and behavior*, Vol. 3, No. 3, pp. 127–37.

Clark, C. E. J., Y. Liu, and H. M. Cooper, 2014, "The Yin and Yang of Wnt/Ryk Axon Guidance in Development and Regeneration", *Science China Life Sciences*, Vol. 57, No. 4, pp. 366–371.

Comas, D., F. Petit, and T. Preat, 2004, "Drosophila Long-Term Memory Formation Involves Regulation of Cathepsin Activity.", *Nature*, Vol. 430, No. 6998, pp. 460–3.

Ding, Z.-Y., Y.-H. Wang, Z.-K. Luo, H.-F. Lee, J. Hwang, C.-T. Chien, and M.-L. Huang, 2011, "Glial Cell Adhesive Molecule Unzipped Mediates Axon Guidance in Drosophila.", *Developmental dynamics: an official publication of the American Association of Anatomists*, Vol. 240, No. 1, pp. 122–34.

Doherty, J., M. A. Logan, O. E. Taşdemir, and M. R. Freeman, 2009, "Ensheathing Glia Function as Phagocytes in the Adult Drosophila Brain.", *The Journal of neuroscience : the official journal of the Society for Neuroscience*, Vol. 29, No. 15, pp. 4768–81.

Edenfeld, G., T. Stork, and C. Klämbt, 2005, "Neuron-Glia Interaction in the Insect Nervous System", *Current Opinion in Neurobiology*, Vol. 15, No. 1, pp. 34–39.

Farris, S. M., 2011, "Are Mushroom Bodies Cerebellum-like Structures?", *Arthropod Structure and Development*, Vol. 40, No. 4, pp. 368–379.

Freeman, M. R., and J. Doherty, 2006, "Glial Cell Biology in Drosophila and Vertebrates", *Trends in Neurosciences*, Vol. 29, No. 2, pp. 82–90.

Fushima, K., and H. Tsujimura, 2007, "Precise Control of Fasciclin II Expression Is Required for Adult Mushroom Body Development in Drosophila", *Development Growth and Differentiation*, Vol. 49, No. 3, pp. 215–227.

Gai, Y., Z. Liu, I. Cervantes-Sandoval, and R. L. Davis, 2016, "Drosophila SLC22A Transporter Is a Memory Suppressor Gene That Influences Cholinergic Neurotransmission to the Mushroom Bodies", *Neuron*, Vol. 90, No. 3, pp. 581–595.

Goodman, C. S., and C. J. Shatz, 1993, "Developmental Mechanisms That Generate Precise Patterns of Neuronal Connectivity", *Cell*, Vol. 72, No. 1001, pp. 77–98.

Goossens, T., Y. Y. Kang, G. Wuytens, P. Zimmermann, Z. Callaerts-végh, G. Pollarolo, R. Islam, M. Hortsch, and P. Callaerts, 2011, "The Drosophila L1CAM Homolog Neuroglian Signals through Distinct Pathways to Control Different Aspects of Mushroom Body Axon Development", Vol. 138, No. 8, pp. 1595–1605.

Grillenzoni, N., A. Flandre, C. Lasbleiz, and J.-M. Dura, 2007, "Respective Roles of the DRL Receptor and Its Ligand WNT5 in Drosophila Mushroom Body Development", *Development*, Vol. 134, No. 17, pp. 3089–3097.

Hakim, Y., S. P. Yaniv, and O. Schuldiner, 2014, "Astrocytes Play a Key Role in Drosophila Mushroom Body Axon Pruning", *PLoS ONE*, Vol. 9, No. 1, pp. 1–12.

Hartenstein, V., 2011, "Morphological Diversity and Development of Glia in Drosophila", *Glia*, Vol. 59, No. 9, pp. 1237–1252.

Heisenberg, M., 2003, "Mushroom Body Memoir: From Maps to Models", *Nature Reviews Neuroscience*, Vol. 4, No. 4, pp. 266–275.

Herculano-Houzel, S., 2012, "The Remarkable, yet Not Extraordinary, Human Brain as a Scaled-up Primate Brain and Its Associated Cost.", *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 109 Suppl, pp. 10661–8.

Hidalgo, a, and G. E. Booth, 2000, "Glia Dictate Pioneer Axon Trajectories in the Drosophila Embryonic CNS.", *Development (Cambridge, England)*, Vol. 127, No. 2, pp. 393–402.

Hitier, R., A. F. Simon, F. Savarit, and T. Préat, 2000, "No-Bridge and Linotte Act Jointly at the Interhemispheric Junction to Build up the Adult Central Brain of Drosophila Melanogaster.", *Mechanisms of development*, Vol. 99, No. 1-2, pp. 93–100.

Hong, S.-T., S. Bang, S. Hyun, J. Kang, K. Jeong, D. Paik, J. Chung, and J. Kim, 2008, "cAMP Signalling in Mushroom Bodies Modulates Temperature Preference Behaviour in Drosophila.", *Nature*, Vol. 454, No. 7205, pp. 771–5.

Inestrosa, N. C., and L. Varela-Nallar, 2015, "Wnt Signalling in Neuronal Differentiation and Development", *Cell and Tissue Research*, Vol. 359, No. 1, pp. 215–223.

Jacobs, J. R., and C. S. Goodman, 1989, "Embryonic Development of Axon Pathways in the Drosophila CNS. II. Behavior of Pioneer Growth Cones.", *The Journal of neuroscience : the official journal of the Society for Neuroscience*, Vol. 9, No. 7, pp. 2412–22.

Joiner, W. J., A. Crocker, B. H. White, and A. Sehgal, 2006, "Sleep in Drosophila Is Regulated by Adult Mushroom Bodies.", *Nature*, Vol. 441, No. 7094, pp. 757–760.

Kaplan, A., C. B. Kent, F. Charron, and A. E. Fournier, 2014, "Switching Responses: Spatial and Temporal Regulators of Axon Guidance", *Molecular Neurobiology*, Vol. 49, No. 2, pp. 1077–1086.

Kolodkin, A. L., and M. Tessier-Lavigne, 2011, "Mechanisms and Molecules of Neuronal Wiring: A Primer", *Cold Spring Harbor Perspectives in Biology*, Vol. 3, No. 6, pp. 1–14.

Kunz, T., K. F. Kraft, G. M. Technau, and R. Urbach, 2012, "Origin of Drosophila Mushroom Body Neuroblasts and Generation of Divergent Embryonic Lineages", *Development*, Vol. 139, No. 14, pp. 2510–2522.

Kurusu, M., T. Awasaki, L. M. Masuda-Nakagawa, H. Kawauchi, K. Ito, and K. Furukubo-Tokunaga, 2002, "Embryonic and Larval Development of the Drosophila Mushroom Bodies: Concentric Layer Subdivisions and the Role of Fasciclin II.", *Development (Cambridge, England)*, Vol. 129, No. 2, pp. 409–419.

Kurusu, M., T. Katsuki, K. Zinn, and E. Suzuki, 2012, "Developmental Changes in Expression, Subcellular Distribution, and Function of Drosophila N-Cadherin, Guided by a Cell-Intrinsic Program during Neuronal Differentiation", *Developmental Biology*, Vol. 366, No. 2, pp. 204–217.

Kurusu, M., T. Nagao, U. Walldorf, S. Flister, W. J. Gehring, and K. Furukubo-Tokunaga, 2000, "Genetic Control of Development of the Mushroom Bodies, the Associative Learning Centers in the Drosophila Brain, by the Eyeless, Twin of Eyeless, and Dachshund Genes.", *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 97, No. 5, pp. 2140–4.

Lakhina, V., C. L. Marcaccio, X. Shao, M. E. Lush, R. a. Jain, E. Fujimoto, J. L. Bonkowsky, M. Granato, and J. a. Raper, 2012, "Netrin/DCC Signaling Guides Olfactory Sensory Axons to Their Correct Location in the Olfactory Bulb", *Journal of Neuroscience*, Vol. 32, No. 13, pp. 4440–4456.

Lee, T., a Lee, and L. Luo, 1999, "Development of the Drosophila Mushroom Bodies: Sequential Generation of Three Distinct Types of Neurons from a Neuroblast.", *Development (Cambridge, England)*, Vol. 126, No. 18, pp. 4065–4076.

Long, H., S. Yoshikawa, and J. B. Thomas, 2016, "Equivalent Activities of Repulsive Axon Guidance Receptors", *Journal of Neuroscience*, Vol. 36, No. 4, pp. 1140–1150.

Mani, R., R. P. St Onge, J. L. Hartman, G. Giaever, and F. P. Roth, 2008, "Defining Genetic Interaction.", *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 105, No. 9, pp. 3461–3466.

Masek, P., and K. Scott, 2010, "Limited Taste Discrimination in Drosophila.", *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 107, No. 33, pp. 14833–8.

Melnattur, K. V., and C. H. Lee, 2011, "Visual Circuit Assembly in Drosophila", *Developmental Neurobiology*, Vol. 71, No. 12, pp. 1286–1296.

Mika, K., 2014, *The Role of Unzipped in Axon Guidance and Targeting in the Olfactory System of Drosophila melanogaster*, MSc Thesis, Bogazici University.

Ng, J., and L. Luo, 2004, "Rho GTPases Regulate Axon Growth through Convergent and Divergent Signaling Pathways.", *Neuron*, Vol. 44, No. 5, pp. 779–93.

Ng, J., T. Nardine, M. Harms, J. Tzu, A. Goldstein, Y. Sun, G. Dietzl, B. J. Dickson, and L. Luo, 2002, "Rac GTPases Control Axon Growth, Guidance and Branching.", *Nature*, Vol. 416, No. 6879, pp. 442–7.

Noveen, A., A. Daniel, and V. Hartenstein, 2000, "Early Development of the Drosophila Mushroom Body: The Roles of Eyeless and Dachshund.", *Development (Cambridge, England)*, Vol. 127, No. 16, pp. 3475–88.

Ou, J., Z. Gao, L. Song, and M. S. Ho, 2016, "Analysis of Glial Distribution in Drosophila Adult Brains", *Neuroscience Bulletin*.

Öztürk, A., 2010, *Characterization of Genes Involved in Photoreceptor Differentiation*, M.Sc. Thesis, Boğaziçi University.

Patapoutian, A., and L. F. Reichardt, 2000, "Roles of Wnt Proteins in Neural Development and Maintenance.", *Current opinion in neurobiology*, Vol. 10, No. 3, pp. 392–9.

Pereanu, W., D. Shy, and V. Hartenstein, 2005, "Morphogenesis and Proliferation of the Larval Brain Glia in Drosophila.", *Developmental biology*, Vol. 283, No. 1, pp. 191–203.

Pollerberg, G. E., K. Thelen, M. O. Theiss, and B. C. Hochlehnert, 2013, "The Role of Cell Adhesion Molecules for Navigating Axons: Density Matters", *Mechanisms of Development*, Vol. 130, No. 6-8, pp. 359–372.

Rangarajan, R., Q. Gong, and U. Gaul, 1999, "Migration and Function of Glia in the Developing Drosophila Eye.", *Development (Cambridge, England)*, Vol. 126, No. 15, pp. 3285–92.

Raper, J., and C. Mason, 2010, "Cellular Strategies of Axonal Pathfinding.", *Cold Spring Harbor perspectives in biology*, Vol. 2, No. 9, pp. 1–22.

Reynaud, E., L. L. Lahaye, A. Boulanger, I. M. Petrova, C. Marquilly, A. Flandre, T. Martianez, M. Privat, J. N. Noordermeer, J. M. Dura, 2015, "Guidance of Drosophila Mushroom Body Axons Depends upon DRL-Wnt Receptor Cleavage in the Brain Dorsomedial Lineage Precursors", *Cell Reports*, Vol. 11, No. 8, pp. 1293–1304.

Seinen, E., J. G. M. Burgerhof, R. C. Jansen, and O. C. M. Sibon, 2011, "RNAi-Induced off-Target Effects in Drosophila Melanogaster: Frequencies and Solutions.", *Briefings in functional genomics*, Vol. 10, No. 4, pp. 206–14.

Sepp, K. J., J. Schulte, and V. J. Auld, 2001, "Peripheral Glia Direct Axon Guidance across the CNS/PNS Transition Zone.", *Developmental biology*, Vol. 238, No. 1, pp. 47–63.

Seugnet, L., Y. Suzuki, G. Merlin, L. Gottschalk, S. P. Duntley, and P. J. Shaw, 2011, "Notch Signaling Modulates Sleep Homeostasis and Learning after Sleep Deprivation in Drosophila.", *Current biology: CB*, Vol. 21, No. 10, pp. 835–40.

Shimizu, K., M. Sato, and T. Tabata, 2011, "The Wnt5/planar Cell Polarity Pathway Regulates Axonal Development of the Drosophila Mushroom Body Neuron.", *The Journal of neuroscience: the official journal of the Society for Neuroscience*, Vol. 31, No. 13, pp. 4944–54.

Siegenthaler, D., E.-M. Enneking, E. Moreno, and J. Pielage, 2015, "L1CAM/Neuroglian Controls the Axon-Axon Interactions Establishing Layered and Lobular Mushroom Body Architecture.", *The Journal of cell biology*, Vol. 208, No. 7, pp. 1003–18.

Simon, A. F., I. Boquet, M. Synguélakis, and T. Préat, 1998, "The Drosophila Putative Kinase Linotte (Derailed) Prevents Central Brain Axons from Converging on a Newly Described Interhemispheric Ring", *Mechanisms of Development*, Vol. 76, No. 1-2, pp. 45–55.

Smith, D. H., 2009, "Stretch Growth of Integrated Axon Tracts: Extremes and Exploitations", *Progress in Neurobiology*, Vol. 89, No. 3, pp. 231–239.

Soldano, A., Z. Okray, P. Janovska, K. Tmejová, E. Reynaud, A. Claeys, J. Yan, Z. K. Atak, B. De Strooper, … B. A. Hassan, 2013, "The Drosophila Homologue of the Amyloid Precursor Protein Is a Conserved Modulator of Wnt PCP Signaling.", *PLoS biology*, Vol. 11, No. 5, pp. e1001562.

Strausfeld, N. J., I. Sinakevitch, S. M. Brown, and S. M. Farris, 2009, "Ground Plan of the Insect Mushroom Body: Functional and Evolutionary Implications", *Journal of Comparative Neurology*, Vol. 513, No. 3, pp. 265–291.

Tamariz, E., and A. Varela-Echavarría, 2015, "The Discovery of the Growth Cone and Its Influence on the Study of Axon Guidance.", *Frontiers in neuroanatomy*, Vol. 9, No. May, pp. 51.

Terzioğlu-Kara, E., 2015, *Elucidating the Role of Unzipped in the Visual System of Drosophila*, PhD. Thesis, Boğaziçi University.

Timofeev, K., W. Joly, D. Hadjieconomou, and I. Salecker, 2012, "Localized Netrins Act as Positional Cues to Control Layer-Specific Targeting of Photoreceptor Axons in Drosophila", *Neuron*, Vol. 75, No. 1, pp. 80–93.

Tucker, E. S., L. A. Oland, and L. P. Tolbert, 2004, "In Vitro Analyses of Interactions between Olfactory Receptor Growth Cones and Glial Cells That Mediate Axon Sorting and Glomerulus Formation.", *The Journal of comparative neurology*, Vol. 472, No. 4, pp. 478–95.

Villegas, S. N., F. A. Poletta, and N. G. Carri, 2003, "GLIA: A Reassessment Based on Novel Data on the Developing and Mature Central Nervous System.", *Cell biology international*, Vol. 27, No. 8, pp. 599–609.

Vitriol, E. A., and J. Q. Zheng, 2012, "Growth Cone Travel in Space and Time: The Cellular Ensemble of Cytoskeleton, Adhesion, and Membrane", *Neuron*, Vol. 73, No. 6, pp. 1068–1080.

Wang, J., C. T. Zugates, I. H. Liang, C. H. J. Lee, and T. Lee, 2002, "Drosophila Dscam Is Required for Divergent Segregation of Sister Branches and Suppresses Ectopic Bifurcation of Axons", *Neuron*, Vol. 33, No. 4, pp. 559–571.

Wang, Y., A.-S. Chiang, S. Xia, T. Kitamoto, T. Tully, and Y. Zhong, 2003, "Blockade of Neurotransmission in Drosophila Mushroom Bodies Impairs Odor Attraction, but Not Repulsion.", *Current biology: CB*, Vol. 13, No. 21, pp. 1900–4.

Weiss, P., 1934, "In Vitro Experiments on the Factors Determining the Course of the Outgrowing Nerve Fiber", *Journal of Experimental Zoology*, Vol. 68, No. 3, pp. 393–448.

Yao, Y., Y. Wu, C. Yin, R. Ozawa, T. Aigaki, R. R. Wouda, J. N. Noordermeer, L. G. Fradkin, and H. Hing, 2007, "Antagonistic Roles of Wnt5 and the Drl Receptor in Patterning the Drosophila Antennal Lobe.", *Nature neuroscience*, Vol. 10, No. 11, pp. 1423–32.

Yu, F., and O. Schuldiner, 2014, "Axon and Dendrite Pruning in Drosophila", *Current Opinion in Neurobiology*, Vol. 27, No. 2, pp. 192–198.

Zhan, X. L., J. C. Clemens, G. Neves, D. Hattori, J. J. Flanagan, T. Hummel, M. L. Vasconcelos, A. Chess, and S. L. Zipursky, 2004, "Analysis of Dscam Diversity in Regulating Axon Guidance in Drosophila Mushroom Bodies", *Neuron*, Vol. 43, No. 12, pp. 673–686.

Zülbahar, S., 2012, *Identification of the Role of a Novel Cell Adhesion Molecule, Unzipped, in Mediating Neuron-Glia Interactions in Drosophila*, M.S. Thesis, Boğaziçi University.

Zwarts, L., F. Van Eijs, and P. Callaerts, 2015, "Glia in Drosophila Behavior", *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology*, Vol. 201, No. 9, pp. 879–893.